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THE LINOLEIC ACID DERIVATIVE DCP-LASHEDS LIGHT ON TYPE 1 DM THERAPY

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Keywords:

DCP-LA, Oral administration, Type 1 DM, Type 2 DM, Therapy

Abstract

The present study investigated the possibility for the linoleic acid derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) as a novel drug of diabetes mellitus (DM). DCP-LA had no effect on intracellulardistribution of the glucose transporter GLUT4 in differentiated 3T3-L1 adipocytes. DCP-LA increased glucose uptake into adipocytes in a bell-shaped concentration (0.01-100 □M)-dependent manner, and the effect was not affected by the PKC inhibitor GF109203X. In the oral glucose tolerance test (OGTT) using type 1 DM model mice treated with streptozotocin and type 2 DM model mice (C57BL/KsJ-leprdb/leprdb mice), intraperitoneal injection with insulin reduced serum glucose levels in a dose (0.2-1.2 x body weight U)-dependent manner. Like insulin, oral administration with DCP-LA effectively reduced serum glucose levels in a dose (0.001-0.1 mg/kg)-dependent manner both in type 1 and type 2 DM model mice. Taken together, these results indicate that DCP-LA is capable ofreducing serum glucose levels by the mechanism independent of that for insulin. DCP-LA, therefore, could be developed as a novel DM drug, especially for type 1 DM.

Introduction

In the process of insulin-induced reduction in serum glucose levels, insulin activates the receptor tyrosine kinase insulin receptor, and the activated insulin receptor phosphorylates its own receptor and insulin receptor substrate 1 (IRS-1) at the tyrosine residues. Activated IRS-1 recruits and activatesphosphoinositide 3-kinase (PI3K), which produces PIP_3 by phosphorylating PIP_2. PIP_3 binds to and activates phosphoinositide-dependent kinase 1 (PDK1). Then, PI3K and/or PDK1 activate Akt [1]. Akt triggers vesicular transport of the glucose transporter GLUT4 towards the cell surface, to increase cell surface localization of GLUT4. GLUT4 on the plasma membrane conveys extracellular glucose into cells, to reduce serum glucose levels [2]. Akt plays a pivotal role in this pathway.

8-[2-(2-Pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) is a linoleic acid derivative with the cyclopropane ring instead of the*cis*-double bond (Figure 1).DCP-LA serves as a selective and direct activator of PKC□ [3,4]. In addition, DCP-LA serves as a strong inhibitor of tyrosine phosphatase 1B (PTP1B)[5]. This raises the possibility that DCP-LA enhances an insulin signaling pathway along an insulin receptor/IRS-1/PI3K/PDK1/Akt axis as a result of persistent tyrosine phosphorylation of insulin receptor and IRS-1 due to PTP1B inhibition. DCP-LA, alternatively, has the potential to activate Akt through direct interaction with PKC□, independently of insulin [6]. This also suggests that DCP-LA might promote GLUT4 translocation towards the cell surface, regardless of an insulin receptor/IRS-1/PI3K/PDK1/Akt pathway.

Then, I postulated that DCP-LA could control serum glucose levels in diabetes mellitus (DM) without insulin. To address this hypothesis, I monitored intracellular GLUT4 distribution and assayed serum glucose levels in the oral glucose tolerance test (OGTT) using type 1 and type 2 DM model mice. I show here that DCP-LA effectively reduces serum glucose levels both in type 1 and type 2 DM model mice by the mechanism independent of that for insulin.

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Materials and methods

Cell culture

3T3-L1-GLUT4myc fibroblast cells, expressing GLUT4myc that is constructed by inserting a human c-MYC epitope (14 amino acids) into the first ectodomain of GLUT4, were cultured and differentiated into adipocytes. We have confirmed in the Oil-Red O staining and Western blot analysis using an anti-peroxisome proliferator-activated receptor antibody that cells used here are well differentiated into 3T3-L1 adipocytes [1].

Monitoring of GLUT4 trafficking

Differentiated 3T3-L1 adipocytes were incubated in Krebs-Ringer-HEPES buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$ and 20mM HEPES, pH 7.5) containing 0.2 % (w/v) bovine serum albumin supplemented with 10 mM glucose for 1 h at 37 °C. Cells were treated with insulin or DCP-LA for 20 min. Then, cells were homogenized by sonication in an ice-cold mitochondrial buffer [210 mM mannitol, 70 mM sucrose, and 1 mM EDTA, 10 mM HEPES, pH 7.5] containing 1% (v/v) protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and subsequently, homogenates were centrifuged at 3,000rpm for 5 min at 4 °C. The supernatants were centrifuged at 11,000rpm for 15 min at 4 °C and further, the collected supernatants were ultracentrifuged at 100,000 *g* for 60 min at 4 °C to separate the cytosolic and plasma membrane fractions. The supernatants and pellets were used as the cytosolic and plasma membrane fractions, respectively. Whether the cytosolic and plasma membrane components were successfully separated was confirmed in the Western blot analysis using antibodies against the cytosolic marker lactate dehydrogenase (LDH) and the plasma membrane marker cadherin. The cytosolic fraction contains GLUT4 in transport vesicles as well as in intracellular compartments such as the endosomes and the trans-Golgi network, and the plasma membrane fraction otherwise contains GLUT4 on the plasma membrane, but not in a partial pool near the plasma membrane.

Protein concentrations for each fraction were determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins in the plasma membrane fraction were resuspended in the mitochondrial buffer containing 1% (w/v) sodium dodecyl sulfate (SDS). Proteins for each fraction were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with TBS-T [150mM NaCl, 0.1% (v/v) Tween-20, and 20mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin (BSA), blotting membranes were reacted with an anti-c-myc antibody (Merck Millipore, Darmstadt, Germany) followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. Immunoreactivity was detected with an ECL kit (Invitrogen, Carlsbad, CA, USA) and visualized using a chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA). Signal density was measured with an ImageQuant software (GE Healthcare).

Glucose uptake assay

Glucose uptake assay was carried out by the method as described previously [1,8,9]. Differentiated 3T3-L1-GLUT4myc adipocytes were incubated in a Krebs-Ringer-HEPES buffer containing 0.2 % (w/v) BSA supplemented with 10 mM glucose at 37 °C for 1 h. Then, cells were treated with DCP-LA in phosphate-buffered saline supplemented with 10 mM glucose at 37 °C for 2 h. After treatment, extracellular solution was collected and glucose was labeled with *p*-aminobenzoic ethyl ester (ABEE), followed by HPLC. Glucose concentration taken up into cells was calculated by subtracting extracellular glucose concentration from initial extracellular glucose concentration (10 mM).

OGTT

OGTT was carried out by the method as described previously [7]. C57BL/6J mice (male, 8 weeks of age)(Japan SLC Inc., Shizuoka, Japan) were intraperitoneally injected with streptozotocin (250 mg/kg) once and used as a type 1 DM model mice 4 days after injection. For normal control group, mice were injected with saline.

C57BL/KsJ-leprdb/leprdb mice are a well-established genetic model of type 2 DM, which have characteristics similar to human type 2 DM including obesity, hyperglycemia, and extreme insulin resistance. C57BL/KsJ-leprdb/leprdb and wild-type C57BL/6J mice (female, 8 weeks) were purchased from CLEA Japan (Tokyo, Japan) and used as a type 2 DM model mice and normal control mice, respectively.

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In OGTT, mice were fasted for 12 h, followed by intraperitoneal injection with insulin or oral administration with DCP-LA using a feeding needle 30 min prior to loading glucose. After collection of blood (10 □L) from the tail vein, the serum was labeled with ABEE and loaded onto the HPLC system and glucose concentrations were calculated from the peak area/concentration calibration curve made before using a standard glucose solution.

Statistical analysis

Statistical analysis was carried out using unpaired *t*-test, analysis of variance (ANOVA) followed by a Bonferroni correction and ANOVA followed by Fisher's protected least significant difference (PLSD) test.

Results

Insulin is recognized to stimulate glucose uptake into cells by promoting GLUT4 translocation towards the cell surface and increasing cell surface localization of GLUT4. DCP-LA(100 nM) had no effect on cell surface localization of GLUT4 in differentiated 3T3-L1 adipocytes (Figure 2). This indicates that DCP-LA stimulates glucose uptake into cells without affectingintracellular GLUT4 distribution.

DCP-LA significantly increased glucose uptake into differentiated 3T3-L1 adipocytes in a bell-shaped concentration (0.01-100 □M)-dependent manner (Figure 3A). DCP-LA-induced increase in the glucose uptake into cells was not affected by the PKC inhibitor GF109203X (100 nM)(Figure 3B). This indicates that DCP-LA stimulates glucose uptake into cells in a PKC-independent manner.

In the OGTT using type 1 DM model mice, intraperitoneal injection with insulin reduced serum glucose levels in a dose (0.2-1.2x body weight unit)-dependent manner, with the significant difference at 0.8 and 1.2x body weight unit (Figure 4A). In the OGTT using type 2 DM model mice, intraperitoneal injection with insulin significantly reduced serum glucose levels at a dose of 1.2x body weight unit (Figure 4B). Similar effects were obtained with DCP-LA too. In the OGTT using type 1 DM model mice, oral administration with DCP-LA reduced serum glucose levels in a dose (0.001-0.1 mg/kg)-dependent manner, with the significant difference at 0.1 mg/kg (Figure 5A). In the OGTT using type 2 DM model mice, oral administration with DCP-LA also reduced serum glucose levels in a dose (0.001-0.1 mg/kg)-dependent manner, with the significant difference at 0.1 mg/kg (Figure 5B). Collectively, these results indicate that DCP-LA exhibits a beneficial effect both on type 1 and type 2 DM model mice.

Discussion

Serum glucose levels are controlled by insulin. Insulin activates insulin receptor, which phosphorylates its own receptor and IRS-1, followed by sequential activation of PI3K, PDK1, and Akt [1]. Activated Akt promotes GLUT4 translocation towards the cell surface and increases cell surface localization of GLUT4, thereby increasing glucose uptake into cells to reduce serum glucose levels [2].Distinct from this pathway, PKC□/□ and PKC□ activate Akt and participate in the regulation of intracellular GLUT4 trafficking and glucose uptake into cells [10].

In the present study, DCP-LA significantly stimulated glucose uptake into differentiated 3T3-L1 adipocytes. Amazingly, DCP-LA had no effect on intracellular GLUT4 distribution in differentiated 3T3-L1 adipocytes. This indicates that DCP-LA stimulates glucose uptake into cells, regardless of GLUT4 translocation through a PI3K/PDK1/Akt pathway. DCP-LA serves a selective and direct activator of PKC□□[3,4]. DCP-LA-induced increase in the glucose uptake into differentiated 3T3-L1 adipocytes was not affected by the PKC inhibitor GF109203X. This rules out the possibility that DCP-LA stimulates glucose uptake into cells by PKC□-mediated Akt activation. It is presently unknown how DCP-LA stimulates glucose uptake into cells. Plausible explanations include that DCP-LA enhances activity of GLUT4 on the plasma membrane in a PKC□-independent manner and that DCP-LA promotes translocation of other glucose transporters except for GLUT4 towards the cell surface. To address this question, we are currently attempting further experiments.

Type 1 DM is caused by lack of insulin in association with disruption of pancreas □ cells, and therefore, insulin injection is indispensable for type 1 DM therapy. A striking finding in the present study is that oral administration with DCP-LA effectively reduced serum glucose levels without insulin in type 1 DM model mice. This raises the possibility that DCP-LA could relieve type 1 DM patients from distress of everyday insulin injection. DCP-LA

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exhibited a beneficial effect on type 2 DM model mice as well, with the potential similar to that for insulin. Overall, these results indicate that DCP-LA could become a promising and novel drug for DM therapy.

Conclusion

The linoleic acid derivative DCP-LA stimulates glucose uptake into differentiated adipocytes without affecting intracellular GLUT4 trafficking and in a PKC-independent manner. DCP-LA effectively reduces serum glucose levels without insulin both in type 1 and type 2 DM model mice. Taken together, these results indicate that DCP-LA cancontrol serum glucose levels by the mechanism independent of that for insulin.

Conflict of interests

I have no conflict of interests.

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Figure 1. Chemical structure of DCP-LA.

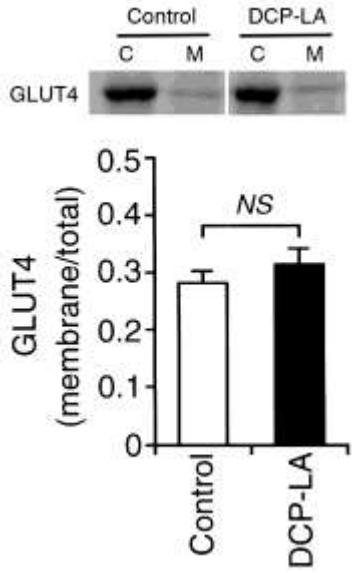

Figure 2. The effect of DCP-LA on intracellular GLUT4 distributionin differentiated 3T3-L1 adipocytes.

Cells were treated with DCP-LA (100 nM) for 20 min. Then, cells were separated into the cytosolic and plasma membrane fractions, followed by Western blotting. In the graph, each column represents the mean (±SEM) signal intensity for GLUT4 on the plasma membrane relative to that for whole cells (n = 4 independent experiments).*NS* (not significant), unpaired *t*-test.

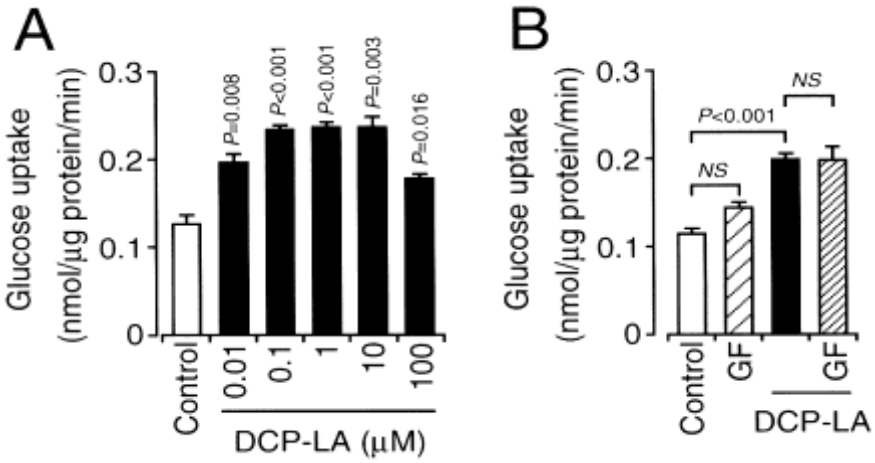

Figure 3.The effect ofDCP-LA on glucose uptake into differentiated 3T3-L1 adipocytes.

Cells were incubated in a glucose (10 mM)-containing extracellular solution for 1 h. Then, cells were treated with DCP-LA at concentrations as indicated (**A**) or DCP-LA at a concentration of 100 nM in the absence and presence of

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GF109203X (GF)(100 nM) (**B**) for 2 h. In the graphs, each column represents the mean (± SEM) glucose uptake (nmol/□g protein/min)(n=4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction.*NS*, not significant.

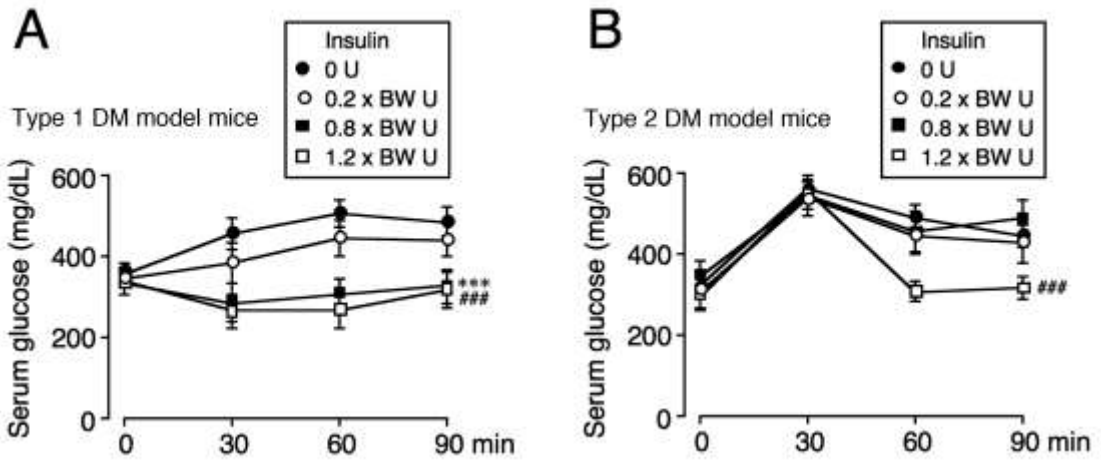

Figure 4.The effect of insulin on serum glucose levels in type 1 and type 2 DM model mice.(A)

OGTT in type 1 DM model mice. Insulinat doses as indicated was intraperitoneally injected into type 1 DM model mice30 min prior to loading glucose (2 g/ml/kg body weight). In the graph, each point represents the mean (± SEM) serum glucose concentration at periods of time as indicated (mg/dL)(n=4 independent mice). ***,###*P<0.001*, ANOVA followed by Fisher's PLSD test. (**B**)OGTT in type 2 DM model mice. Insulin at doses as indicated was intraperitoneally injected into type 2 DM model mice 30 min prior to loading glucose (2 g/ml/kg body weight). In the graphs, each point represents the mean (± SEM) serum glucose concentration (mg/dL)(n=6-8 independent mice). ###*P<0.001*, ANOVA followed by Fisher's PLSD test.

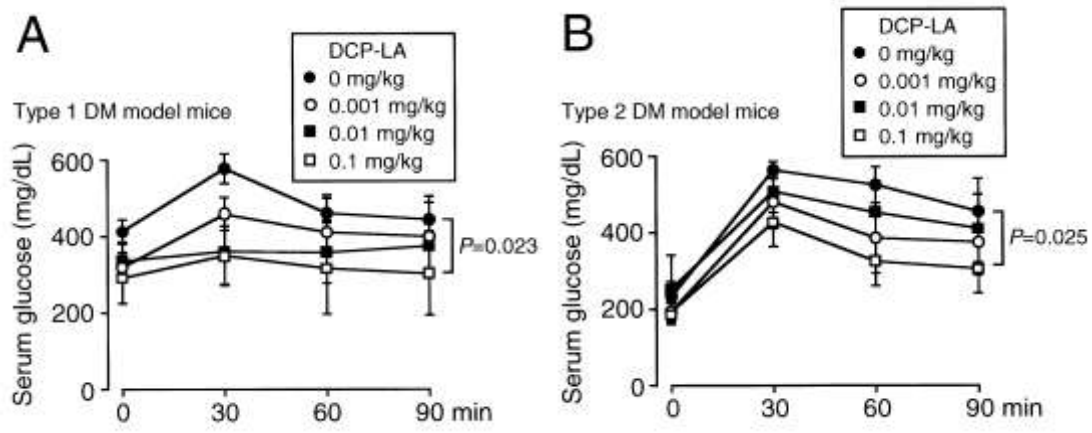

Figure 4.The effect of DCP-LA on serum glucose levels in type 1 and type 2 DM model mice.(A)

OGTT in type 1 DM model mice. DCP-LAat doses as indicated was orally administered to type 1 DM model mice 30 min prior to loading glucose (2 g/ml/kg body weight). In the graph, each point represents the mean (± SEM) serum glucose concentration at periods of time as indicated (mg/dL)(n=4 independent mice). *P* value, ANOVA followed by Fisher's PLSD test. (**B**)OGTT in type 2 DM model mice. DCP-LAat doses as indicated was orally administered to type 2 DM model mice 30 min prior to loading glucose (2 g/ml/kg body weight). In the graphs,

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each point represents the mean (± SEM) serum glucose concentration (mg/dL)(n=7 independent mice). *P* value, ANOVA followed by Fisher's PLSD test.