THE PHOSPHATIDYLETHANOLAMINE DERIVATIVE diDCP-LA-PE STIMULATES VESIVULAR GLUT4 EXOCYTOSIS BY INTERACTING WITH NSF

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Abstract

We have earlier found that the phosphatidylethanolamine derivative ,1,2-O-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-sn-glycero-3 phosphatidylethanolamine (diDCP-LA-PE) promotes translocation of the glucose transporter GLUT4 towards the cell surface and facilitates glucose uptake into cells. The present study was conducted to understand the role of N-ethylmaleimide-sensitive factor (NSF) in the diDCP-LA-PE-induced GLUT4 trafficking. Like insulin, diDCP-LA-PE increased cell surface localization of GLUT4 in differentiated 3T3-L1 adipocytes, and the effect was abolished by the vesicular exocytosis inhibitorbotulinum toxin-A. diDCP-LA-PE-induced GLUT4 translocation is clearly inhibited by knocking-down NSF. These results indicate that diDCP-LA-PE stimulates vesicular GLUT4 exocytosis by interacting with NSF.

Introduction

We have earlier synthesized the phosphatidylethanolamine derivative 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylethanolamine (diDCP-LA-PE). diDCP-LA-PE contains the linoleic acid derivative8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) at the α and β position on phosphatidylethanolamine (Figure 1). diDCP-LA-PEhas the potential to inhibit protein tyrosine phosphatase 1B (PTP1B) and to activate protein kinase C (PKC) ε, -ι, and -ζ [1]. diDCP-LA-PE indirectly activates Akt through a pathway along an insulin receptor substrate 1 (IRS-1)/phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase-1 (PDK1) axis in association with PTP1B inhibition [2]. diDCP-LA-PE, alternatively, activates Akt by direct interaction with PKCε and PKCζ/ι [2]. Then, activated Akt triggers translocation of the glucose transporter GLUT4 towards the cell surface, to convey extracellular glucose into cells and reduce serum glucose levels [2]. Moreover, the Rho GTPase Rac1 and Rho-associated coiled-coil-containing protein kinase (ROCK) are implicated in diDCP-LA-PE-induced GLUT4 translocation [3]. It is well-recognized that the small G proteins including Rac1 connect the motor protein myosin V and the cargo, bearing vesicular transport.

N-ethylmaleimide-sensitive factor (NSF), an ATPase, is a member to regulate vesicular traffic [4]. NSF associates with the NSF adaptor soluble NSF attachment protein (SNAP) [5] and SNAP receptors (SNAREs) such as syntaxin, SNAP25 and synaptobrevin [6]. The vesicular SNARE synaptobrevin, which connects to cargo-containing transport vesicle, assembles the target SNAREs syntaxin and SNAP25, and in turn, SNAP binds to the SNARE assembly, followed by NSF binding. Then, a complex of vesicle/SNAREs/SNAP/NSF is dissociated and the vesicle fuses into the membrane, leading to vesicular exocytosis. This prompted me to examine the role of NSF in diDCP-LA-PE-induced GLUT4 translocation towards the cell surface.

I show here that NSF participates in diDCP-LA-PE-induced GLUT4 vesicular transport.

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 [7].

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 a variety of lipids in the presence and absence of inhibitors 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).

NSF knockdown
The siRNA to silence the NSF-targeted gene(Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the negative control (NC) siRNA, which has the scrambled sequence with the GC content and nucleic acid composition same as that for the NSF siRNA (Ambion, Carlsbad, CA, USA), were transfected into differentiated 3T3-L1-GLUT4myc adipocytes using a Lipofectamine reagent, and cells were used for experiments 48 h after transfection.

Whether NSF is successfully knocked down was confirmed in the western blotting analysis using antibodies against NSF (Cell Signaling Technology, Inc., Danvers, MA, USA) and □-actin (SIGMA, Missouri, SL, USA).

Statistical analysis
Statistical analysis was carried out using analysis of variance (ANOVA) followed by a Bonferroni correction and unpaired t-test.

Results and discussion
Insulin (100 nM) significantly increased cell surface localization of GLUT4 in differentiated 3T3-L1 adipocytes (Figure 1). Like insulin, diDCP-LA-PE (1 □M) also increased the cell surface localization of GLUT4, to an extent similar to that for insulin (not significant between the insulin effect and the diDCP-LA-PE effect, ANOVA followed

by a Bonferroni correction)(Figure 2). This indicates that diDCP-LA-PE promotes GLUT4 translocation towards the cell surface.
diDCP-LA-PE-induced increase in the cell surface localization of GLUT4 was abolished by 24-h pretreatment with the vesicular exocytosis inhibitor botulinum toxin-A (BoTX)(0.1 U/ml)(Figure 3). This indicates that diDCP-LA-PE increases the cell surface localization of GLUT4 by stimulating vesicular exocytosis of GLUT4.

Expression of NSF was significantly decreased in differentiated 3T3-L1 adipocytes transfected with the siRNA to silence the NSF-targeted gene as compared with that in cells transfected with the NC siRNA (Figure 4A). This confirms that NSF is successfully knocked-down in adipocytes used here. diDCP-LA-PE-induced increase in the cell surface localization of GLUT4 was clearly inhibited by knocking-down NSF (Figure 4B). Taken together, these results indicate that NSF is required for diDCP-LA-PE-induced vesicular exocytosis of GLUT4.

NSF is well-recognized to play a central role in the vesicular release of neurotransmitters at presynaptic terminals [8].Interestingly, NSF also regulates vesicular transport of neurotransmitter receptors such as AMPA receptor, $GABA_A$ receptor, $GABA_B$ receptor, β_2-adrenergic receptor, D_1 and D_2 dopamine receptors, muscarinic M_1, M_3, M_4 and M_5 ACh receptors, and □7 nicotinic ACh receptor[9-20]. NSF forms a complex with SNAP, the vesicular SNARE synaptobrevin, and the target SNAREs syntaxin and SNAP25. NSF produces high-energy phosphates by ATP hydrolysis, andthe complex of vesicle/SNAREs/SNAP/NSF is dissociated using the energy. Then, vesicle (cargo)containing neurotransmitters or presenting receptors on the membrane is released, moves towards the cell surface, and fuses into the plasma membrane for vesicular exocytosis.

In the present study, diDCP-LA-PE-induced increase in the cell surface localization of GLUT4 in differentiated 3TL1 adipocytes is clearly inhibited by the vesicular exocytosis inhibitor BoTX and by knocking-down NSF. This provides evidence that diDCP-LA-PE promotes vesicular exocytosis of GLUT4 in an NSF-dependent manner. It is presently unknown whether SNAREs expressed in neurons are also expressed in adipocytes. To address this question, further experiments need to be carried out.

Conclusion
The results of the present study show thatdiDCP-LA-PE stimulates vesicular exocytosis by interacting with NSF in adipocytes, thereby increasing cell surface localization of GLUT4. This may extend our understanding the mechanism for diDCP-LA-PE-regulated GLUT4 trafficking.

Conflict of interests
I have no conflict of interests.

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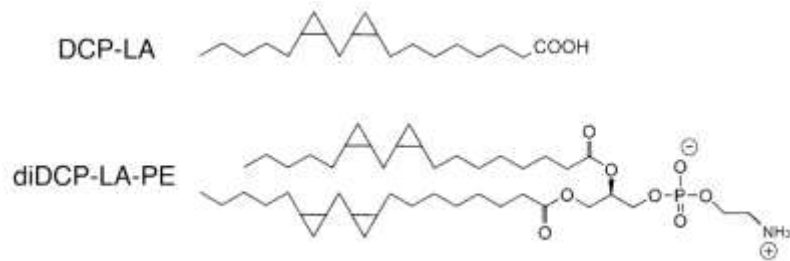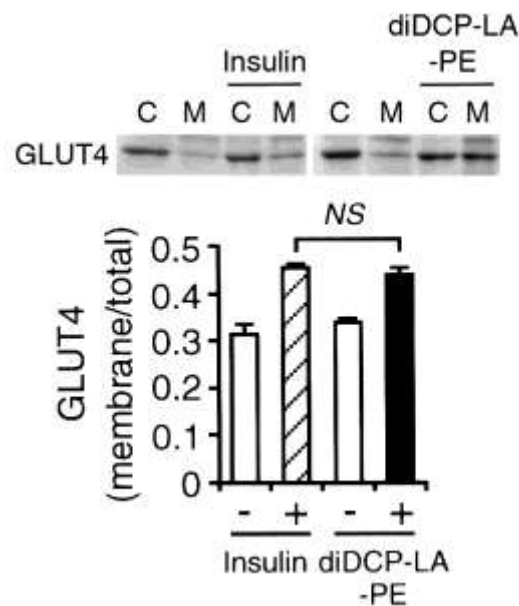
Figure 1. Chemical structure of diDCP-LA-PE.

Figure 2.*diDCP-LA-PE increases cell surface localization of GLUT4 in differentiated 3T3-L1 adipocytes.*

Adipocytes were treated with insulin (100 nM) or diDCP-LA-PE (1 □M)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), ANOVA followed by a Bonferroni correction.
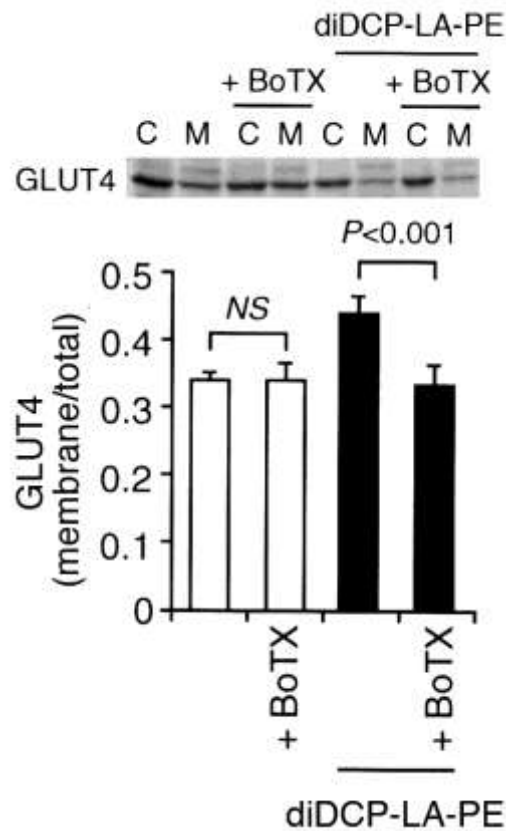
Figure 3.diDCP-LA-PE increases cell surface localization of GLUT4 by stimulating vesicular exocytosis of GLUT4 in differentiated 3T3-L1 adipocytes.

Adipocytes were pretreated and untreated with BoTX (0.1 U/ml) for 24 h prior to treatment with diDCP-LA-PE (1 □M)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. *P* value, ANOVA followed by a Bonferroni correction.
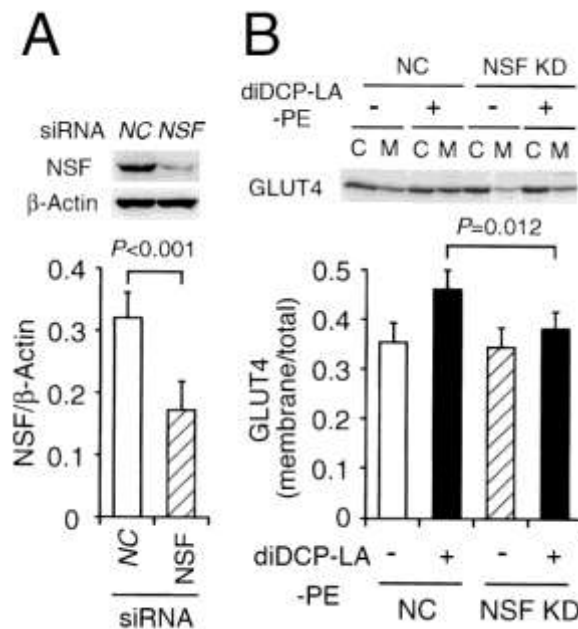
Figure 4.diDCP-LA-PE stimulates vesicular exocytosis of GLUT4 by interacting with NSF in differentiated 3T3-L1 adipocytes.

(**A**) Adipocytesweretransfectedwith siRNAs forNCand NSF, and 48 h later Western blotting was carried out. The signal intensity for GLUT4 protein was normalized by that for □-actin. In the graphs, each column represents the mean (±SEM) normalized intensity (n = 4 independent experiments). *P* value, unpaired *t*-test.

(**B**) Adipocytes transfected with the NC and NSF siRNAs were treated with diDCP-LA-PE (1 □M)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. KD, knocked-down.*P* value, ANOVA followed by a Bonferroni correction.