LRRK2 Inhibition Ameliorates Dexamethasone-Induced Glucose Intolerance via Prevents Impairment in GLUT4 Membrane Translocation in Adipocytes

Motoki Imai,⁎ a,b Fumitaka Kawakami, a,b Makoto Kubo, b,c Makoto Kanzaki, d Hiroko Maruyama, b,e Rei Kawashima, a,b Tatsunori Maekawa, a,b Yoshifumi Kurosaki, b,f Fumiaki Kojima, b,g and Takafumi Ichikawa⁎, a,b

⁎Department of Regulation Biochemistry, Graduate School of Medical Sciences, Kitasato University; Sagamihara 252–0373, Japan; a Research Facility of Regenerative Medicine and Cell Design, Kitasato University School of Allied Health Science; Sagamihara 252–0373, Japan; b Department of Clinical Immunology, Graduate School of Medical Sciences, Kitasato University; Sagamihara 252–0373, Japan; c Department of Biomedical Engineering, Graduate School of Biomedical Engineering, Tohoku University; Sendai 980–8579, Japan; d Department of Cytopathology, Graduate School of Medical Sciences, Kitasato University; Sagamihara 252–0373, Japan; e Department of Medical Laboratory Sciences, Kitasato University School of Allied Health Sciences; Sagamihara 252–0373, Japan; and f Department of Pharmacology, Kitasato University School of Allied Health Sciences; Sagamihara 252–0373, Japan.

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are associated with Parkinson’s disease. LRRK2 is a large protein with multiple functional domains, including a guanosine 5′-triphosphate (GTP)-binding domain and a protein kinase domain. Recent studies indicated that the members of the Rab GTPase family, Rab8a and Rab10, which are involved in the membrane transport of the glucose transporter type 4 (GLUT4) during insulin-dependent glucose uptake, are phosphorylated by LRRK2. However, the physiological role of LRRK2 in the regulation of glucose metabolism is largely unknown. In the present study, we investigated the role of LRRK2 using dexamethasone (DEX)-induced glucose intolerance in mice. LRRK2 knockout (KO) mice exhibited suppressed glucose intolerance, even after treatment with DEX. The phosphorylation of LRRK2, Rab8a and Rab10 was increased in the adipose tissues of DEX-treated wild-type mice. In addition, inhibition of the LRRK2 kinase activity prevented the DEX-induced inhibition of GLUT4 membrane translocation and glucose uptake in cultured 3T3-L1 adipocytes. These results suggest that LRRK2 plays an important role in glucose metabolism in adipose tissues.

Key words leucine-rich repeat kinase 2 (LRRK2); glucose transporter 4 (GLUT4); adipocyte; glucose intolerance; dexamethasone

INTRODUCTION

The leucine-rich repeat kinase 2 (LRRK2) gene encodes a large protein kinase harboring multiple functional domains, including guanosine 5′-triphosphate (GTP)-binding and kinase domains.1–3) Several missense mutations in LRRK2 have been reported; approximately 10% of autosomal dominant familial Parkinson’s disease (PD) patients carry a mutation in LRRK2. Furthermore, 3.6% of sporadic PD patients have a mutation in LRRK2. Of >50 mutations in the LRRK2 gene reported to date, the G2019S point mutation is the most common pathogenic mutation.5) This mutation is known to increase LRRK2 kinase activity, subsequently inducing mitochondrial dysfunction and neuronal cell death.6,7) Recently, LRRK2 kinase activity was observed to be increased in patients with idiopathic PD even in the absence of LRRK2 mutations.8) Thus, abnormal activation of LRRK2 kinase activity may critically contribute to the pathogenesis of PD; therefore, LRRK2 may be a potential therapeutic target for a broader population of idiopathic and familial PD.

LRRK2 is expressed in multiple organs, including the brain, kidney, lung, and immune cells. In our preliminary studies, we found that LRRK2 is highly expressed in adipose.

Although the adipose tissue is a major site for storing and mobilizing energy, obesity-induced lipid accumulation and hypertrophy of adipocytes lead to insulin resistance and the development of type 2 diabetes.9–11) Recent studies indicated that PD patients with LRRK2 mutations showed higher prediabetes rates and that diabetes mellitus may increase the risk of PD.12) Furthermore, it has been reported that glucose intolerance with a high-fat diet exacerbates in MitoPark mice, which are PD model mice, and it has been suggested that the causative genes for PD may be associated with abnormal glucose metabolism.13) In addition, Yu et al. demonstrated that LRRK2 is expressed in 3T3-L1 mouse preadipocytes and regulates lipid storage in differentiated 3T3-L1 adipocytes via the phosphorylation of Rab8a.

Rab GTPases, such as Rab8a and Rab10, have been reported to be required for the insulin-dependent translocation of glucose transporter type 4 (GLUT4) to the plasma membrane (PM) in insulin-sensitive cells, such as adipocytes and muscle cells.15) Previous studies suggested that the phosphorylation of Rab by LRRK2 inhibits its interaction with the guanosine 5′-diphosphate (GDP) dissociation inhibitor, resulting in the conversion of Rab to the active GTP-bound form.16) These results suggest that LRRK2 negatively regu-
lates glucose uptake in adipocyte and muscle cells by suppressing GLUT4 membrane translocation via the phosphorylation-dependent inactivation of Rab8a and Rab10 signaling. Therefore, LRRK2 may function as a negative regulator of glucose metabolism. Moreover, overactivation of LRRK2 kinase activity is presumed to lead to abnormality of glucose metabolism in peripheral tissues. And also, we hypothesized that LRRK2 kinase activity is a novel therapeutic target for glucose metabolism abnormalities in peripheral tissues.

Glucocorticoids are steroid hormones that are secreted from the adrenal medulla and are essential for maintaining biological functions.16,17 However, they also function as stress hormones and previous studies suggested that their levels in the blood are increased due to mental and physical stress and aging; furthermore, chronic elevation of glucocorticoid levels can lead to abnormal glucose metabolism.18,19 Dexamethasone (DEX) is a synthetic glucocorticoid with a wide range of therapeutic uses because of its anti-inflammatory and immunosuppressive properties. However, its main side effect, i.e., impaired glucose tolerance and reduced insulin sensitivity in vulnerable patients, depending on the dose and frequency of administration, may lead to diabetes.20 In addition, it has been used in previous studies to induce insulin resistance in animal models.21 Here, we investigated the effect of LRRK2 on GLUT4 membrane translocation and glucose tolerance using models of DEX-induced glucose intolerance in both mice and cultured adipocytes.

MATERIALS AND METHODS

Animal Experiments C57BL/6J male wild-type (WT) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Congenic LRRK2-knockout (KO) mice on the C57BL/6J background were kindly supplied by Dr. Matthew J Farrer (University of Florida, U.S.A.).22 The animal experiments described here were approved by the Animal Experiment Committee of Kitasato University School of Medical Hygiene (Approval number Eiken-ken 18-09-3) and the Genetic Modification Experiment Safety Committee (Approval No. 4454).

WT and LRRK2-KO mice (age, 8–9 weeks) were used in this study. Mice were allowed access to chow and water ad libitum. Mice were injected daily with a standardized dose of DEX (2mg/kg, D4902, Sigma-Aldrich, MO, U.S.A.) via intraperitoneal injection for 7 d. DEX was dissolved in 100% ethanol and diluted 100 times with phosphate-buffered saline (PBS) before use. The same volume of 100% ethanol and PBS were injected into the negative control group. The animals were classified into four experimental groups: WT mice injected with PBS and ethanol (WT), WT mice injected with DEX (WT-DEX), KO mice injected with PBS (KO), and KO mice injected with DEX (KO-DEX). These mice were bred and maintained at room temperature (22°C) with a 12 h light/dark cycle in the conventional room of the Kitasato University school of Medical Hygiene and Animal Experiment Facility, with two or three animals per cage.

Intraperitoneal Glucose Tolerance Test (ipGTT) Intraperitoneal glucose tolerance tests (ipGTT) were performed after the 7-d DEX treatments. Mice were subjected to fasting for 12–14 h prior to the ipGTT. Blood was collected from the tail vein of the mouse and the fasting blood glucose level was measured using a glucometer (Glutest Neo Sensor; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). Subsequently, a 20% glucose solution was injected intraperitoneally at a dose of 2g/kg of body weight.

Blood was collected from the tail vein 15, 30, 60, 90, and 120 min after the glucose injection, and the blood glucose level was measured as described above. Glucose tolerance evaluation in the ipGTT was performed based on the area under the curve (AUC) of the change in blood glucose concentration after intraperitoneal injection of 20% glucose.

Cell Culture and Induction of Insulin Resistance Pre-adipocyte cells 3T3-L1 cells and myc-GLUT4-ECFP stably expressing 3T3-L1-G4 cells, established by Dr. Makoto Kanazaki were cultured in low-glucose (1000 mg/L) Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, GIBCO, Thermo Fisher Scientific, MA, U.S.A.), 100 µg/mL penicillin (Nacalai Tesque, Kyoto, Japan), and 100 µg/mL streptomycin (Nacalai Tesque) at 37°C in a humidified atmosphere with 5% CO₂.

For the differentiation of adipocytes, cells were supplemented with high-glucose (4500 mg/L) DMEM with 10% FBS until a confluence of 90% was reached, after which the differentiation of 3T3-L1 preadipocytes to adipocytes was initiated using differentiation medium (MDI induction medium) containing 1 µg/mL insulin (Sigma-Aldrich I-5500), 0.5 mmol/L isobutylmethylxanthine (Sigma-Aldrich I-7018), and 0.1 µmol/L DEX (Sigma-Aldrich D-4902) for 48 h. Subsequently, the medium was replaced with DMEM containing 1 µg/mL insulin (insulin medium) for 48 h and 3T3-L1 cells were cultured in DMEM with 10% FBS for 48–72 h, until the occurrence of differentiation. After differentiation, insulin resistance was induced in 3T3-L1 adipocytes via treatment with 2 µM DEX (DEX was dissolved in 100% ethanol to 2 mM and stored), and the same volume of 100% ethanol was delivered to the negative control group. Cells were treated with selective LRRK2 inhibitors (CZC25146 or MLi-2), whereas the negative control group was treated with the same volume of dimethyl sulfoxide (DMSO). The two LRRK2 inhibitors were stocked at 1 and 2 mM (CZC25146) or 0.1 and 0.2 mM (MLi-2) in DMSO. Finally, 3T3-L1 adipocytes were used Immunofluorescent cytochemistry, Western blotting or glucose uptake test.

Western Blot Analysis Cultured cell pellets and epididymal adipose tissue samples collected from experimental mice were homogenized on ice in radio immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)) supplemented with protease inhibitors and phosphatase inhibitors (HALT protease and phosphatase inhibitor cocktail 100×, Thermo Fisher Scientific) and 0.5 M ethylenediaminetetraacetic acid (EDTA) (0.5 M EDTA solution 100×, Thermo Fisher Scientific). Subsequently, homogenates were sonicated on ice and these cell and tissue lysates were centrifuged at 18000×g for 20 min at 4°C. Protein concentration in the extracts was determined using a protein assay (Pre-Diluted Protein Assay Standards BSA Set, Thermo Fisher Scientific). Samples were then supplemented with NuPage® LDS sample buffer 4× (NP0008, Invitrogen, Thermo Fisher Scientific) and boiled for 5 min at 100°C, and 15 µg (cultured cells; PM) or 30 µg (cultured cells; total, tissue samples) of protein was run on e-PAGE 5–20% gradient gels (ATTO, Tokyo, Japan). The separated proteins in the gel were transferred to a poly-
vinylidene difluoride (PVDF) membrane (Merck Millipore; MA, U.S.A.) on a Bio-Rad Trans-Blot Turbo transfer system. The membrane was then blocked in PVDF Blocking Reagent for Can Get Signal (TOYOBO, Osaka, Japan) and incubated overnight with the primary antibody in Can Get Signal solution 1 (TOYOBO) at 4°C. Blots were washed 3 times with 0.05% Tween-20-supplemented tris-buffered saline (TBS-T) and incubated with a horseradish-peroxidase-conjugated (HRP) or fluorescence-conjugated secondary antibody in Can Get Signal solution 2 (TOYOBO) for 1 h at room temperature. Membranes were washed again 3 times with TBS-T and bands were visualized using enhanced chemiluminescence (Price ECL Plus Substrate, Thermo Fisher Scientific) or detected by fluorescence imaging on an ONSDEY Fe imaging system (Li-COR, U.S.A.). To normalize the signal of phospho-specific antibodies to the target protein, strips were stripped via incubation with stripping buffer (EzReprobe, ATTO) for 30 min at 40°C, followed by washing steps in TBS-T and blocking in PVDF Blocking Reagent. Blots were then incubated with an antibody against the total target protein. Band signals were quantitatively analyzed using the Image Studio software (LI-COR). All phospho-specific antibodies were used at a dilution of 1:1000 and total antibodies were used at a dilution of 1:2000. Antibodies against the following proteins were used: GLUT4 (2213S; Cell Signaling Technology, MA, U.S.A.), AKT (9272S; Cell Signaling Technology), P-AKT(S473, 4051S; Cell Signaling Technology), Rab10 (11808-I-AP; Proteintech), P-Rab10 (T73, ab23026; Abcam), Rab8a (ab237702; Abcam), P-Rab8a (T73, ab230260; Abcam), Rab10 (11808-I-AP; Proteintech), Insulin receptor substrate 1 (IRS-1) (#3407; Cell Signaling Technology), P-IRS-1 (S307, 2381T; Cell Signaling Technology), LRRK2 (ab033474; Abcam, Cambridge, U.K.), P-LRRK2 (S935, ab133450; Abcam), Rab8a (ab237702; Abcam), P-Rab8a (T72, ab230260; Abcam), Rab10 (11808-I-AP; Proteintech), Proteintech, Insulin receptor substrate 1 (IRS-1) (#3407; Cell Signaling Technology), P-IRS-1 (S307, 2381T; Cell Signaling Technology), LRRK2 (ab033474; Abcam, Cambridge, U.K.), P-LRRK2 (S935, ab133450; Abcam), Rab8a (ab237702; Abcam), P-Rab8a (T72, ab230260; Abcam), Rab10 (11808-I-AP; Proteintech), Proteintech, Insulin receptor substrate 1 (IRS-1) (#3407; Cell Signaling Technology), P-IRS-1 (S307, 2381T; Cell Signaling Technology), IR (#3025; Cell Signaling Technology), E-cadherin (sc-15000-20; Santa Cruz Biotechnology, TX, U.S.A.) and β-actin (5125S; Cell Signaling Technology, BA3R; Invitrogen). The secondary antibodies were HRP donkey anti-mouse immunoglobulin G (IgG) (H + L) antibody (Jackson ImmunoResearch, PA, U.S.A.), HRP donkey anti-rabbit IgG (H + L) antibody (Jackson Immuno Research) and HRP donkey anti-goat IgG antibody (Santa Cruz Biotechnology), all at a dilution of 1:5000.

Immunofluorescent Cytochemistry (ICC) and Fluorescent Detection of 3T3-L1-G4 Cells Preadipocyte cells expressing myc-GLUT4-ECFP stably (3T3-L1-G4 cells) were cultured and differentiated in 24-well plates, then transferred onto glass-bottom dishes (No. 1S thickness, 0.16–0.19 mm; Matsumani Glass, Kishiwada, Japan) coated with laminin. After differentiation, cells were cultured with DEX for 24h and then cells were treated with LRRK2 inhibitors for 12h. Cells were incubated in Krebs Ringer Phosphate N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (KRPH) buffer for 20min, stimulated with insulin for 10min, fixed with 4% paraformaldehyde in PBS for 10min, and blocked for 60min in 1% BSA in PBS at room temperature. An anti-myc primary antibody (017-21871; Wako Pure Chemical Corporation, Ltd., Osaka, Japan) at 1:300 dilution in blocking solution was added to the wells, followed by incubation overnight at 4°C. After washing, a secondary antibody (Alexa 594-labeled anti-mouse IgG, Cell Signaling Technology) at a dilution of 1:500 in blocking solution was used to detect the myc epitope of myc-GLUT4-ECFP at the cell surface. Finally, cells were washed by distilled water and mounted for visualization and obtain fluorescent images of stained cells using confocal fluorescence microscope (C2 Si; Nikon, Tokyo, Japan). Pixel intensity of single cells (25–30 cells per condition, each repeated in 3 independent experiments) was quantified by Image software (National Institutes of Health, Bethesda, MD, U.S.A.).

PM Protein Isolation 3T3-L1 cells were seeded in 6-well plates and differentiated into adipocytes. After differentiation, cells were cultured in the presence of DEX for 24h and then treated with LRRK2 inhibitors (2 µM CZC25146 and 0.2 µM MLi-2) for 12h. Cells were incubated in KRPH buffer for 20 min and then stimulated with 150nM insulin for 12min. After insulin stimulation, cells were washed with cold PBS and harvested. The PM fraction was isolated using a Minute PM Protein Isolation Kit (Invent Biotechnologies, Eden Prairie, MN, U.S.A.) according to the manufacturer’s instructions. All steps were performed at 4°C. Briefly, cultured cells were lysed in buffer A and placed in a filter cartridge. After centrifugation at 16000 × g for 30s, pellets were centrifuged at 700 × g for 1min. The supernatant was collected and centrifuged at 16000 × g for 15min. The supernatant was then collected as the cytosol protein fraction and the pellet as the total membrane fraction, which was resuspended in buffer B and centrifuged at 7800 × g for 5min. The supernatant was then centrifuged at 16000 × g for 25min, and the pellet was collected as the PM protein fraction for Western blot analysis.

Measurement of Glucose Uptake in 3T3-L1 Cells Glucose uptake in 3T3-L1 cells was determined using a glucose cellular uptake measurement kit (COSMO bio Co., Ltd.), according to the manufacturer’s instructions. Briefly, 3T3-L1 cells were seeded and differentiated in 12-well plates, followed by the induction of insulin resistance and treatment with LRRK2 inhibitors (2 µM CZC25146 and 0.2 µM MLi-2) for 24h, respectively. After the removal of the culture medium, cells were incubated in serum-free medium for 6h and washed with KRPH buffer containing 1% BSA. Subsequently, the cells were stimulated with 150nM insulin and incubated for 10min after the addition of a 1mM 2-deoxyglucose solution. Cells were then washed with cold PBS, collected in sample diluent buffer, and immediately sonicated. Cell lysates were heated at 80°C for 15min and immediately centrifuged (15000 × g for 20min). Supernatants were mixed in a 96-well black plate in a reaction solution that contained a fluorescent substrate and an enzyme (diaphorase) and incubated at 37°C for 2h in the dark. The fluorescence intensity of the samples was measured on a fluorescence plate reader.

Statistical Analysis Data were analyzed using the GraphPad prism 8 software (GraphPad Software). All experimental data were expressed relative to control values and are presented as means ± standard deviation (S.D.). p-Values were calculated using Tukey’s test or student’s t test and significance was set at p < 0.05.

RESULTS Dexamethasone-Induced Insulin Resistance Was Suppressed in LRRK2-KO Mice In this study, we induced insulin resistance via intraperitoneal administration of DEX to WT or KO mice. Under this experimental condition, we observed no significant differences in weight after DEX ad-
ministration between WT and KO mice in our experimental conditions (Supplementary Figs. 1A, B). Moreover, no differences were observed in food and drinking-water consumption between WT and KO mice (Supplementary Figs. 1C, D). Subsequently, glucose tolerance was evaluated using an ipGTT. In WT mice, the AUC was significantly increased by DEX administration compared with the control group, whereas no effect was observed in KO mice. Furthermore, the AUC in DEX-injected KO mice was significantly lower than that detected in DEX-injected WT mice (Figs. 1A, B). These results suggest that DEX injection induces glucose intolerance in WT mice, but not in KO mice.

**DEX Administration Suppressed the Activation and Expression of Glucose-Uptake-Related Molecules in the Adipose Tissues of LRRK2-KO Mice**

Insulin-sensitive tissues, such as adipose tissues, skeletal muscles, and the liver, play a central role in the maintenance of blood glucose levels through insulin-dependent glucose uptake. Our preliminary experiments showed that LRRK2 is highly expressed in adipose tissues. Therefore, we analyzed the expression and phosphorylation of LRRK2 and insulin-signal-related molecules in adipose tissues by Western blotting. DEX injection did not significantly affect the expression of GR, IR, and P-IRS-1 (Ser307) in either WT and KO mice (Figs. 2A, 2B, 2C). However, the phosphorylation of AKT (Ser473) was significantly decreased in DEX-injected WT mice, but not in DEX-injected KO mice (Fig. 2D). Similarly, GLUT4 expression was significantly decreased by DEX injection in WT mice, but not in KO mice (Fig. 2E). These results suggest that LRRK2 suppress the phosphorylation of AKT (Ser473) and the expression of GLUT4 in adipose tissues in the presence of DEX.

Next, we assessed the expression and phosphorylation of LRRK2, Rab8a, and Rab10 in these mice. DEX injection significantly increased the phosphorylation of LRRK2 (S935), whereas the expression of total LRRK2 remained unchanged, by DEX in WT mice (Fig. 2F). The phosphorylation of Rab8a and Rab10 was significantly increased by DEX injection in WT mice (Figs. 2G, H). In contrast, phosphorylated Rab8a and Rab10 were hardly detected in KO mice, which confirmed that the phosphorylation of these two Rab GTPases was caused by LRRK2. These results suggest that DEX increases the kinase activity of LRRK2 in adipose tissues.

**LRRK2 Inhibition Reduced the Suppression of GLUT4 Membrane Translocation and Glucose Uptake Induced by DEX**

Upon insulin stimulation, GLUT4 translocates from the cytoplasm to the cell membrane, to transport glucose into cells. However, it has been reported that glucose uptake is inhibited by the suppression of GLUT4 membrane translocation in conditions of insulin resistance. Therefore, to investigate the role of LRRK2 in the translocation of GLUT4 to the cell membrane in DEX-induced insulin resistance conditions, the effects of DEX and selective LRRK2 kinase inhibitors on GLUT4 membrane translocation were assessed using 3T3-L1 cells expressing myc-GLUT4-ECFP stably (3T3-L1-G4 cells). First, we confirmed the presence of insulin resistance after DEX administration to cells and found that DEX suppressed the insulin-stimulated GLUT4 membrane translocation compared with control cells (Figs. 3A, B). Under these conditions, the translocation of GLUT4 to the cell membrane was recovered by two LRRK2 kinase inhibitors, CZC25146 and MLi-2 (Figs. 3A, B) Furthermore, located GLUT4 at plasma membrane (PM) was detected by Western blotting. As a result, CZC25146 and MLi-2 restored translocated GLUT4 (Fig. 4A). At this time, there was no difference in the total GLUT4 (Fig. 4B). Moreover, intracellular glucose uptake was significantly reduced by DEX compared with the control (Fig. 5). In contrast, treatment with CZC25146 and MLi-2 improved the decreased glucose uptake caused by DEX (Fig. 5). These results suggest that the inhibition of LRRK2 kinase activity ameliorates the DEX-induced decline in glucose uptake observed in adipocytes.

**DISCUSSION**

GLUT4 plays an important role in the metabolic homeostasis by regulation of glucose uptake in insulin-sensitive tissues, such as adipose and muscle. In response to insulin
signal, GLUT4 translocates to the PM from cytoplasm via a signal cascade involving various molecules such as IRS-1 and AKT.28–30 As a result, extracellular glucose uptake is increased and blood glucose levels are reduced.31 Recently, Rab GTPases, including Rab3, Rab8, Rab10, Rab35, and Rab7L1, were identified as substrates of LRRK2.14 Notably, Rab8a and Rab10 act as important regulators of GLUT4 membrane trafficking. Rab10 directly promotes the translocation of GLUT4-containing vesicles to the membrane and its docking at the PM in adipocytes.32 Rab8a colocalize with GLUT4 in adipocyte and involved in GLUT4 recycling through the endosomal system after GLUT4 membrane translocation. Recently, it is reported that LRRK2 is expressed in 3T3-L1 adipocyte and that LRRK2-mediated phosphorylation of Rab8a promote lipid storage in adipocyte.11,33 Therefore, we investigated physiological role of LRRK2 in the insulin signaling pathway, including Rab GTPase-mediated GLUT4 vesicle trafficking in adipose tissues using DEX-induced glucose intolerance model.

Here, we found that DEX treatment induced glucose intolerance in WT mice (Fig. 1) and DEX treatment reduced GLUT4 expression and Akt phosphorylation in the adipose tissues of WT mice (Fig. 2D). In cultured adipocytes, DEX treatment reduced glucose uptake and GLUT4 membrane translocation (Figs. 3, 4, 5). In contrast, these effects were reversed by knockout of LRRK2 in mice and by LRRK2 kinase inhibitors in the cells. In addition, we observed that no significant changes of serum insulin level in DEX treated or untreated LRRK2-KO mice, whereas the insulin level was significantly elevated by DEX treatment in WT mice (Supplementary Figs. 2A, B). These results suggest that DEX-induced glucose intolerance is caused by LRRK2-associated mechanisms, indicating that LRRK2 may play an important role in the regulation of blood glucose levels through the modulation of glucose uptake by GLUT4 in adipose tissues.

By Western blotting analysis, we found that DEX treatment significantly decreased Akt phosphorylation at Ser473 in WT mice, but not in LRRK2-KO mice (Fig. 2D). Several kinases that phosphorylate Akt have been reported. 3-Phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates Akt Thr308 at the cell membrane and partially activates Akt. Complete activation of Akt enzymatic activity is caused by mTORC2-mediated phosphorylation of Akt at Ser473. Conversely, Akt is dephosphorylated by protein phosphatase 2A (PP2A) and PH-domain leucine-rich-repeat-containing protein phosphatase 1/2 (PHLP1/2). Interestingly, previous stud-
ies have suggested that DEX increases PP2A activity in the mouse liver; PP2A has been identified as an interacting partner of LRRK2. In addition, LRRK2 has been reported to bind directly to Akt. Therefore, we hypothesized that DEX may induce the formation of a tripartite complex including LRRK2, Akt, and PP2A, thus resulting in the dephosphorylation of Akt in the adipose tissues of DEX-treated mice.

It is known that activation of Akt upregulates glucose uptake and GLUT4 gene expression in adipocytes. In this study, we found that DEX treatment suppressed not only Akt phosphorylation but also GLUT4 expression in the adipose tissues of WT mice. In contrast, LRRK2-KO suppressed the DEX-induced downregulation of GLUT4. These results suggest that the DEX-mediated decrease of Akt phosphorylation causes the depletion of GLUT4 expression in adipose tissues, and that the inhibition of LRRK2 may restore the DEX-induced GLUT4 depletion through prevention of reduced Akt phosphorylation. In addition to this, inflammatory cytokines have been reported to decrease GLUT4 expression in adipose tissues with or without DEX treatment. In WT mice, the mRNA expression of tumor necrosis factor (TNF)-α was significantly increased in adipose tissues (Supplementary Fig. 3A), suggesting that the long-term administration of DEX induces the expression of TNF-α and thereby decreases the expression of GLUT4. Although DEX is an anti-inflammatory drug, previous studies demonstrated that its long-term administration increases glucocorticoid receptor beta (GRβ) expression and the suppression of GR nuclear translocation during a process termed glucocorticoid resistance. Furthermore, it was reported that glucocorticoid resistance induced by the long-term administration of DEX upregulates the production of inflammatory cytokines in neuronal cells. Taken together, our results suggest that the long-term administration of DEX induces TNF-α production in adipocytes, resulting in the downregulation of GLUT4 expression. On the other hand, in LRRK2-KO mice, TNF-α expression was induced by the long-term administration of DEX, whereas no changes in GLUT4 expression were observed. These results suggest that LRRK2 might be involved in the mechanism underlying the suppression of GLUT4 expression mediated by TNF-α signaling.

The Western blotting analysis also showed that the LRRK2 kinase activity toward phosphorylation and Rab GTPases (Rab8a and Rab10) was significantly elevated in the adipose tissues of DEX-treated WT mice (Figs. 2F–H). In the previous studies, it was demonstrated that activation of toll-like receptor 4 (TLR4) promotes LRRK2 kinase activity in microglia and that TLR signaling-activated inhibitor of nuclear factor kappaB (IkB) kinase increases the phosphorylation of LRRK2 at Ser935 in macrophages. In fact, expression of TLR4 mRNA was increased in adipose tissue of DEX-treated WT mice (Supplementary Fig. 3C). Therefore, it was considered that DEX treatment may induce the LRRK2 kinase activation through TLR4 signaling in adipose tissue. In addition, TLR4 expression has been reported to be downregulated by Akt.

Fig. 3. Effect of LRRK2 Inhibition on GLUT4 Membrane Translocation in DEX-Induced Insulin-Resistant 3T3-L1 Adipocyte Cells

Representative image showing cell surface GLUT4 (Myc; red) and total GLUT4 (ECFP; blue). Adipocyte 3T3-L1 cells expressing Myc-GLUT4-ECFP stably (3T3-L1-G4 cells) were treated with (A) control (ethanol + DMSO), dexamethasone (2µM + DMSO), dexamethasone (2µM) + CZC25146 (1 or 2µM), or dexamethasone (2µM) + MLI-2 (0.1 or 0.2µM). After stimulation with 100nM insulin for 10min, 3T3-L1-G4 cells were fixed with 4% PFA. Cell surface GLUT4 was detected with anti-myc antibody followed by an Alexa594-labeled secondary antibody. (B) Shows the ratio of cell surface GLUT4 to total GLUT4. Data presented are representative microscopic images and mean ± S.D. of about 25–30 myc and ECFP-positive cells (total 75–90) in each group from three independent experiments. The data were analyzed by one-way ANOVA combined with Tukey's post hoc test. **p < 0.01, ***p < 0.001, ****p < 0.0001. Scale bar, 100 µm.
In the present study, we found that AKT phosphorylation was decreased in the adipose tissue of DEX-treated WT mice. Thus, DEX treatment may upregulate TLR4 expression via the downregulation of AKT in adipose tissue. Collectively, our results suggest that the long-term administration of DEX upregulates TLR4 expression by suppressing AKT and results in the stimulation of LRRK2 kinase activity by TLR4 signaling in adipose tissue. Thus, increased LRRK2 kinase activity in the adipose tissue of DEX-treated mice may inhibit GLUT4 membrane trafficking by enhancing Rab phosphorylation, subsequently decreasing glucose uptake via GLUT4 and inducing insulin resistance.

In vivo, it was found that DEX treatment-induced glucose intolerance was improved in LRRK2-KO mice. From this, we expect that LRRK2 may negatively regulate glucose uptake and GLUT4 membrane translocation in adipocytes. To confirm this, we examined the effect of DEX on GLUT4 membrane translocation and glucose uptake using stable 3T3-L1 adipocytes expressing myc-GLUT4-ECFP. Expectedly, the membrane translocation of GLUT4 and glucose uptake was significantly suppressed by the exposure of cultured adipocytes to DEX (Figs. 3–5). In addition, we found that LRRK2 kinase inhibitors restored the DEX-induced decrease in GLUT4 membrane translocation and glucose uptake in cultured adipocytes (Figs. 3–5). Thus, LRRK2 kinase activity is required for the DEX-induced negative regulation of the GLUT4-dependent glucose uptake in adipocytes.

Recent studies suggest that Rab GTPase, involved in vesicle trafficking, is a substrate of LRRK2. Phosphorylation of Rab GTPase inhibits the interaction with the guanine-nucleotide exchange factors (GEF) that binds when it is converted to GTP-bound form, which is the active form suggesting that LRRK2 acts as a regulator of vesicular trafficking through...
the phosphorylation of Rab GTPase. Collectively, the present study indicates that LRRK2 kinase activity negatively regulates Rab GTPase-mediated GLUT4 membrane translocation in adipose tissue, therefore abnormal LRRK2 kinase activation may cause impaired glucose tolerance. However, recently, Funk et al. reported that the insulin-induced GLUT4 membrane transport was diminished in LRRK2-deficient rat fibroblasts, and that the phosphorylation of Rab10 was significantly decreased by insulin stimulation in WT fibroblasts, whereas no phosphorylation of Rab10 was observed in LRRK2-KO cells. Thus, those authors concluded that LRRK2 promotes insulin-dependent GLUT4 membrane transport via the phosphorylation of Rab10. This discrepancy suggests that LRRK2 may exert cell-type specific action on the Rab-mediated GLUT4 membrane trafficking.

Glucose uptake and gluconeogenesis in the muscle and liver also play an important role in the regulation of glucose tolerance. We found that the mRNA expression of genes involved in gluconeogenesis, such as PEPCK and G6Pase, was decreased by insulin-dependent GLUT4 membrane transport via the phosphorylation of Rab10. This discrepancy suggests that LRRK2 may exert cell-type specific action on the Rab-mediated GLUT4 membrane trafficking.

Finally, epidemiological studies show that PD patients have abnormalities in glucose metabolism and that type-2 diabetes is linked to an increased risk of PD. Furthermore, it was reported that PD patients with the LRRK2 G2019S kinase activation mutation had a higher diabetic morbidity rate compared to idiopathic PD and that non-manifesting carriers (NMC) with a high probability for future PD also showed increased rates of prediabetes. Therefore, we predict that glucose intolerance induced by the abnormal activation of LRRK2 may be associated with PD pathogenesis.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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