

A Novel Xanthene Derivative, DS20060511, Attenuates Glucose Intolerance by Inducing Skeletal Muscle-specific GLUT4 Translocation in Diabetic Mice

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Abstract

Reduced glucose uptake into the skeletal muscle is an important pathophysiological abnormality in type 2 diabetes, and is caused by impaired translocation of glucose transporter 4 (GLUT4) to the skeletal muscle cell surface. We found a novel xanthene compound, DS20060511, which induces GLUT4 translocation to the skeletal muscle cell surface, thereby stimulating glucose uptake into the skeletal muscle. DS20060511 induced GLUT4 translocation and glucose uptake into differentiated L6-miyotubes and into the skeletal muscles of live mice. These effects were completely abolished in GLUT4 knockout mice. Induction of GLUT4 surface translocation by DS20060511 was independent of the insulin signaling pathways including IRS1-Akt-AS160 phosphorylation and IRS1-Rac1-actin polymerization, eNOS pathway and AMPK pathway. Acute and chronic DS20060511 treatment attenuated the glucose intolerance in obese diabetic mice. Taken together, DS20060511 acts as a skeletal muscle specific-GLUT4 translocation enhancer to facilitate glucose utilization. Further studies with DS20060511 would help to develop a novel antidiabetic medicine.

Introduction

Glucose transporter 4 (GLUT4), which is one of the glucose transporter isoforms that is expressed in the skeletal muscle, myocardium, and adipose tissue, is the rate-limiting transporter for glucose uptake and plays a crucial role in the maintenance of glucose homeostasis^{1,2}. Subjects with type 2 diabetes show reduced glucose uptake by the skeletal muscle because of impaired GLUT4 translocation to the skeletal muscle cell surface³. It has been reported that GLUT4-overexpressing diabetic mice show markedly reduced plasma glucose levels under both fasting and postprandial conditions⁴⁻⁶.

Although GLUT4 is stored in intracellular storage vesicles under basal conditions, insulin induces translocation of GLUT4 to the cell surface, facilitating glucose uptake^{7,8}. Insulin activates Akt via insulin receptor substrate (IRS)-phosphoinositide 3-kinase (PI3K)^{9,10}, and the activated Akt phosphorylates and consequently inhibits the proteins Akt substrate of 160 kDa (AS160) and TBC1 domain family member 1 (TBC1D1), both of which are Rab GTPase-activating proteins (GAPs); this results in activation of the Rab proteins and translocation of GLUT4 to the plasma membrane surface¹¹. RAS-related C3 botulinum toxin substrate 1 (Rac1), another molecule downstream of PI3K, has been reported to promote GLUT4 translocation independently of the Akt-AS160/TBC1D1-Rab pathway. Rac1 stimulates reorganization of the cortical actin polymerization, which allows the GLUT4-containing vesicles to be inserted into the plasma membrane^{12,13}. Insulin is known to regulate GLUT4 translocation via both the Akt-AS160-Rab pathway and Rac1-actin polymerization pathway^{14,15}. In subjects with type 2 diabetes, both the insulin signaling pathways are impaired in the skeletal muscle, resulting in a reduction of insulin-induced glucose uptake by the skeletal muscle.

Contraction during exercise is another important enhancer of GLUT4 translocation in the skeletal muscle^{16,17}. Upon increased glucose demand during exercise in the skeletal muscle, GLUT4 translocates

to the cell surface to promote glucose supply to the skeletal muscle^{18,19}. Exercise increases the AMP/ATP ratio caused by ATP consumption, leading to AMP-activated kinase (AMPK) activation. Activated AMPK stimulates GLUT4 translocation by phosphorylation-inhibition of TBC1D1, which results in activation of the Rab proteins^{20,21}. Furthermore, AICAR, which induces AMPK activation, was shown to increase glucose uptake by the skeletal muscle. Skeletal muscle contraction did not induce phosphorylation of IRS1 or PI3K²². Contraction-induced glucose uptake or GLUT4 translocation was not inhibited by wortmannin, a PI3K inhibitor, in the skeletal muscle^{23,24}. Moreover, combination of insulin and skeletal muscle contraction caused a further increase of GLUT4 translocation and glucose uptake as compared to insulin alone²⁴. These data suggest that skeletal muscle contraction stimulates GLUT4 translocation independently of insulin.

In subjects with type 2 diabetes, skeletal muscle biopsy specimens obtained during euglycemic insulin clamp showed impaired insulin signaling in the skeletal muscle²⁵. Other studies have demonstrated reduced GLUT4 translocation and glucose uptake in subjects with type 2 diabetes^{3,25}. Furthermore, it was reported that the reduced GLUT4 translocation in subjects with type 2 diabetes was improved by exercise^{26,27}. These findings suggest that induction of GLUT4 translocation in the skeletal muscle could be a potential therapeutic target in patients with type 2 diabetes.

In the present study, we screened our chemical libraries using L6-GLUT4myc myotubes and identified a novel xanthene compound, DS20060511, a skeletal muscle cell-specific GLUT4 translocation enhancer. Treatment with DS20060511 decreased the blood glucose level by increasing skeletal muscle glucose uptake via inducing GLUT4 translocation. This glucose-lowering effect of DS20060511 was completely abolished in GLUT4 knockout (GLUT4KO) mice. Treatment with DS20060511 increased the glucose uptake even further when combined with insulin and exercise. GLUT4 translocation by DS20060511 was independent of the insulin receptor (IR) pathways including the IRS1-Akt-AS160 phosphorylation and IRS1-Rac1-actin polymerization, the endothelial nitric oxide synthase (eNOS) pathway and the AMPK pathway. Both acute and chronic DS20060511 treatment attenuated the glucose intolerance in obese diabetic mice. These data suggest that DS20060511 increases glucose uptake into the skeletal muscle via inducing GLUT4 translocation.

Results

The xanthene derivative DS20060511 is a skeletal muscle cell-specific inducer of GLUT4 translocation

We screened our chemical libraries using L6-GLUT4myc myotubes to identify a compound that would induce translocation of GLUT4 to the cell surface in the skeletal muscle cells, and hit upon one compound. Derivatization of the hit compound finally yielded the more potent xanthene compound, DS20060511 (Fig. 1a). Treatment with DS20060511 increased GLUT4 translocation in differentiated L6-GLUT4myc myotubes in a concentration-dependent manner, as is the case with insulin treatment (Fig. 1b

left panel). However, while insulin treatment also increased GLUT4 translocation in differentiated 3T3-L1-GLUT4myc adipocytes, DS20060511 treatment had almost no effect on GLUT4 translocation in these adipocytes, suggesting that the induction of GLUT4 translocation by DS20060511 is specific to skeletal muscle cells (Fig. 1b right panel). Consistent with these data, DS20060511 treatment significantly increased 2-DG uptake in a concentration-dependent manner in L6-GLUT4myc myotubes, as is the case with insulin treatment (Fig. 1c left panel). Again, while insulin was shown to increase 2-DG uptake in differentiated 3T3-L1-GLUT4myc adipocytes, DS20060511 showed no such effect in the adipocytes (Fig. 1c right panel). These data suggest that the novel xanthene compound DS20060511 promotes glucose uptake by skeletal muscle cell-specific activation of GLUT4 translocation.

Treatment with DS20060511 decreases blood glucose levels by increasing skeletal muscle glucose uptake via inducing enhanced GLUT4 translocation in vivo

To investigate whether DS20060511 treatment modulates the glucose dynamics in vivo, DS20060511 was administered orally to normal chow-fed mice. Pretreatment with DS20060511 produced a dose-dependent decrease of the blood glucose levels during the oral glucose tolerance test (GTT) (Fig. 2a). Insulin secretion during oral GTT was rather significantly decreased in all the DS20060511-treated groups, suggesting that DS20060511 treatment decreases blood glucose levels independently of insulin secretion. DS20060511 treatment produced a significant increase in the uptake of [³H]-2-DG in the soleus and gastrocnemius muscles, but not in heart or white adipose tissue (WAT) during intraperitoneal GTT (Fig. 2b). Western blot analysis revealed increased GLUT4 protein expression levels in the plasma membrane fraction of the skeletal muscles in the DS20060511-treated group as seen in an insulin-treated group (Fig. 2c). These data suggest that DS20060511 treatment decreases the blood glucose levels by increasing skeletal muscle glucose uptake via inducing GLUT4 translocation in vivo.

The glucose-lowering effect of DS20060511 is dependent on GLUT4

To confirm that the glucose-lowering effect of DS20060511 is mediated by GLUT4, we administered DS20060511 to GLUT4KO mice. GLUT4 protein expression was undetectable in the skeletal muscle, heart, and WAT of the GLUT4KO mice (Supplementary Fig. 1). While DS20060511 treatment caused a significant decrease of the blood glucose and plasma insulin levels in the wild-type (WT) mice during oral GTT, these effects were completely abolished in the GLUT4KO mice (Fig. 3a). DS20060511 treatment significantly increased the 2-DG uptake by isolated soleus and extensor digitorum longus (EDL) muscles of the WT mice, whereas no such increase in muscle uptake was observed in the isolated muscles of the GLUT4KO mice treated with DS20060511 (Fig. 3b). These data confirm that the glucose-lowering effect of DS20060511 is mediated by GLUT4 in the skeletal muscle.

Treatment with DS20060511 induces GLUT4 translocation without activation of the IR-IRS1-PI3K-Akt-AS160 and -PI3K-Rac1 pathways

The insulin-induced GLUT4 translocation are activated by 1) the IR-IRS1-PI3K-Akt-AS160 pathway, and 2) the IR-IRS1-PI3K-Rac1 pathway in the skeletal muscle. Insulin binds the IR, which results in the activation of IRS1, PI3K and Akt. Activated Akt inhibits the Rab GTPase-activating protein (GAP) AS160, which results in activation of the Rab proteins and translocation of GLUT4 to the plasma membrane²⁸. On the other hand, Rac1 is activated by PI3K and promotes actin remodeling, resulting in translocation of GLUT4¹². We examined whether DS20060511 treatment increases GLUT4 translocation in the skeletal muscle via these pathways. Although the IR β -subunit and IRS1 were phosphorylated in the skeletal muscles of the insulin-treated mice, no such phosphorylation of these proteins was observed after DS20060511 treatment (Fig. 4a). Similarly, while insulin treatment significantly induced phosphorylation of Akt and AS160, DS20060511 treatment had no such effect (Fig. 4b). We next performed immunofluorescence microscopy to investigate whether DS20060511 treatment promotes actin polymerization. Although cell surface GLUT4 was strongly stained by both insulin and DS20060511 treatment, actin polymerization was observed only following insulin treatment in the differentiated L6-GLUT4myc myotubes (Fig. 4c). Moreover, although GLUT4 translocation was induced by both insulin and DS20060511 treatment, Latrunculin B, an actin polymerization inhibitor, only suppressed GLUT4 translocation induced by insulin, but not that induced by DS20060511 treatment (Fig. 4d). Co-treatment of DS20060511 and insulin resulted in an additive increase of GLUT4 translocation in the L6-GLUT4myc myotubes, even at the insulin concentration at which GLUT4 translocation by insulin alone was saturated (Fig. 4e). Consistent with these data, 2-DG uptake induced by insulin was also additively increased by concomitant treatment with DS20060511 in isolated skeletal muscles (Fig. 4f). In fact, blood glucose levels were reduced to a greater degree after combined DS20060511 plus insulin treatment as compared to that after insulin treatment alone in streptozotocin (STZ)-treated mice (Fig. 4g). These data suggest that activation of the IR-IRS1-PI3K-Akt-AS160 and -PI3K-Rac1 pathways is not involved in the GLUT 4 translocation induced by DS20060511 treatment.

Treatment with DS20060511 increases glucose uptake without AMPK activation during exercise

Since exercise, like insulin, is well-known to enhance GLUT4 translocation and increase glucose uptake into the skeletal muscle¹⁷, we next investigated the effect of DS20060511 treatment during exercise. The respiratory quotient (RQ) was significantly increased during running exercise, suggesting that DS20060511 treatment during exercise increased glucose utilization (Fig. 5a). In fact, glucose oxidation was significantly increased during running exercise, whereas fat oxidation was significantly decreased (Fig. 5b, c). 2DG uptake was elevated to a greater degree following electrical muscle stimulation combined with DS20060511 treatment as compared that following electrical muscle stimulation alone without DS20060511 treatment (Fig. 5d). Since electrical muscle stimulation and exercise have been

reported to increase GLUT4 translocation by activation of AMPK²⁹, we investigated the phosphorylation of AMPK by Western blotting following DS20060511 treatment. Although the AMPK phosphorylation level was elevated by electrical muscle stimulation, no such change was observed after DS20060511 treatment (Fig. 5e). The AMPK phosphorylation levels in the skeletal muscles remained unchanged after DS20060511 treatment, as compared to that before the treatment, in vivo (Fig. 5f). These data suggest that the increase in glucose uptake induced by DS20060511 is independent of AMPK activation.

Treatment with DS20060511 decreases the blood glucose in an eNOS-independent manner

It has been shown that sodium nitroprusside (SNP), a nitric oxide (NO) donor, increases glucose uptake in the skeletal muscle and that this increase is not inhibited by the PI3K inhibitor, wortmannin³⁰. In addition, exercise-induced glucose uptake by the skeletal muscle was not suppressed by the NO inhibitor N^G-monomethyl-L-arginine (L-NMMA)³⁰. These data suggest that NO induces glucose uptake by the skeletal muscle via a mechanism that is distinct from both the insulin and exercise signaling pathways. Endothelial nitric oxide synthase, which is a major enzyme generating NO, is expressed in the skeletal muscle. Glucose uptake has been reported to be impaired in isolated skeletal muscles from eNOSKO mice³¹. To investigate the mechanism underlying the increase in glucose uptake by the skeletal muscle induced by DS20060511, we administrated DS20060511 to eNOSKO mice. Treatment with DS20060511 significantly decreased blood glucose levels in both the WT and eNOSKO mice during oral GTT (Fig. 6a, b). Although the blood glucose levels were reduced by insulin treatment, the blood glucose levels were reduced even further after DS20060511 treatment, in both the WT and eNOSKO mice, similarly (Fig. 6c, d). These data suggest that the glucose-lowering effect of DS20060511 is exerted in an eNOS-independent manner.

Acute and chronic DS20060511 treatment improves glucose intolerance in obese diabetic mice

To investigate whether DS20060511 treatment can attenuate glucose intolerance in obese diabetic mice, we conducted oral GTT in high-fat diet (HFD)-fed mice after DS20060511 treatment. Treatment with DS20060511 significantly decreased the blood glucose levels in the HFD-fed mice to the same levels as those observed in normal-chow diet-fed mice during oral GTT (Fig. 7a). The plasma insulin levels were rather decreased in the DS20060511-treated HFD-fed mice (Fig. 7a). Suppression of insulin-induced 2-DG uptake in isolated skeletal muscle from HFD-fed mice was completely restored by DS20060511 treatment (Fig. 7b). These data suggest that acute DS20060511 treatment improves glucose intolerance in obese diabetic mice. Next, we investigated the effect of chronic DS20060511 treatment in genetically obese diabetic (*db/db*) mice. The blood glucose levels decreased significantly from the first to the 28th day of DS20060511 treatment in the *db/db* mice (Fig. 7c, d). Consistent with these data, the hemoglobin A1c (HbA1c) value was also significantly reduced after chronic DS20060511 treatment (Fig. 7e). There were no statistically significant differences in the body weight, food intake, fasting blood glucose level, or fasting plasma insulin levels between the DS20060511- and vehicle-treated *db/db* mice (Supplementary

Fig. 2). These data suggest that both acute and chronic DS20060511 treatment improves diabetes via restoring impaired skeletal muscle glucose uptake.

Discussion

We explored our chemical libraries using L6-GLUT4myc myotubes for a new drug to treat type 2 diabetes, and discovered the novel xanthene compound, DS20060511. DS20060511 increased GLUT4 translocation in differentiated L6-GLUT4myc myotubes, but not in undifferentiated L6-GLUT4myc myotubes, undifferentiated 3T3-L1-GLUT4myc adipocytes, or differentiated 3T3-L1-GLUT4myc adipocytes, suggesting that it acts primarily in the skeletal muscles. Consistently, in vivo, DS20060511 induced 2-DG uptake in the soleus and gastrocnemius muscles, but not in the heart or adipose tissue. Insulin promotes glucose uptake in the adipose tissue as well as skeletal muscle, which inevitably leads to obesity. However, DS20060511 enhances glucose uptake only in the skeletal muscle, and therefore does not cause obesity, making it potentially effective also for prevention of obesity. In fact, DS20060511 reduced the blood glucose levels in obese diabetic mice, without causing hyperphagia, body weight gain, or hypoglycemia, and without increase of the insulin secretion. These characteristics could be preferable to a safe and effective drug for the treatment of type 2 diabetes.

The glucose-lowering effect of DS20060511 was completely abolished in GLUT4KO mice, indicating that DS20060511 increases glucose uptake in a GLUT4-dependent manner. Interestingly, DS20060511 failed to activate upstream insulin signaling including phosphorylation of AS160 and actin remodeling or the AMPK pathway, which are also known to increase GLUT4 translocation in the skeletal muscle. Moreover, DS20060511 enhanced glucose uptake further when combined with insulin and exercise. Thus, DS20060511 may act via an anti-diabetic agent, based on a totally novel mechanism, for patients with impaired skeletal insulin action and those receiving insulin and/or exercise therapy for both type 1 and 2 diabetes.

Some compounds have also been previously reported to induce GLUT4 translocation. Novel pyridazine compounds were reported to strongly induce GLUT4 translocation in L6-myotubes and to show a significant blood glucose-lowering effect in a mouse model of severe diabetes³². Proton uncouplers, such as 2,4-dinitrophenol, are well known to induce GLUT4 translocation in accordance with a rapid drop in the intracellular ATP levels³³. However, unlike DS20060511, these compounds promote GLUT4 translocation via the PI3K or AMPK pathway. The NO-donating small molecule NCX 4016 has been reported to induce GLUT4 translocation in 3T3-L1 adipocytes, but not in skeletal muscle cells³⁴. These findings suggest that a skeletal muscle-specific GLUT4 translocation enhancer like DS20060511 has never previously been reported.

Why does DS2006051 act selectively on the skeletal muscle? The target molecule of DS2006051 may be selectively expressed in the skeletal muscle. The amount of GLUT4 on the cell surface is determined by the balance between exocytosis from the intracellular storage vesicles and endocytosis from the cell membrane. DS2006051 may promote exocytosis or suppress endocytosis of GLUT4 via target molecule

activation. To investigate the selective target molecule of DS20060511 in the skeletal muscle and L6 myotubes, we used three different approaches; radiolabeled compound binding, compound-immobilized beads purification, and UV photo-crosslinking of a compound to the target. Radiolabeled or chemically-modified compounds were allowed to react with samples prepared from skeletal muscle tissue or L6-GLUT4myc myotubes, such as lysates, microsomes, or living cells. After enrichment and purification matched for each approach, the samples were analyzed by LC-MS/MS. Unfortunately, we could not identify any specific target molecule that bound to DS20060511 with a high affinity (data not shown), and further investigation is needed to identify the molecular target of DS20060511.

In conclusion, we identified a novel xanthene compound, DS20060511, and demonstrated that treatment with DS20060511 induced GLUT4 translocation independently of canonical insulin signaling and AMPK activity, to enhance glucose uptake by the skeletal muscle. Moreover, DS20060511 treatment also ameliorated glucose intolerance in obese diabetic mice. Although we could not identify the specific target molecule of DS20060511 on the skeletal muscle cell, further studies with the compound would help to develop a novel drug for type 2 diabetes.

Methods

Chemicals

DS20060511 (Fig. 1a, molecular formula $C_{30}H_{32}O_6 \cdot C_4H_{11}N$, molecular weight 561.71) was obtained from the Medicinal Chemistry Research Laboratories, Daiichi Sankyo Co., Ltd.

2-Deoxy-D-[1,2- 3H]-glucose ($[^3H]$ -2-DG, ART0103A) and D-[1- ^{12}C]-mannitol ($[^{12}C]$ -mannitol, ARC0127A) were purchased from American Radiolabeled Chemicals.

Animals

C57BL/6 and *db/db* mice, as well as CE-2 normal chow and high-fat diet 32 (HFD) were purchased from CLEA Japan. GLUT4 knockout mice were generated at RIKEN BioResource Center according to the previous report³⁵. The mice were group-housed under a 12-h light-dark cycle and given free access to normal chow and water. The HFD-fed obese mice were prepared by feeding C57BL/6 mice a HFD for at least 8 weeks from 6 weeks of age. Male mice were used for all the experiments in this study. The animal care and experimental procedures used in the study were approved by The University of Tokyo Animal Care Committee, and the study was carried out in accordance with the Animal Experimentation Guidelines of Daiichi-Sankyo Co., Ltd.

Streptozotocin (STZ)-treated C57BL/6 mouse model

C57BL/6 mice deprived of access to food overnight received an intraperitoneal injection of STZ (125 mg/kg) and a repeat injection a week later. Thereafter, their blood glucose levels were monitored and insufficient mice (blood glucose levels of under 400 mg/dl and blood glucose levels after overnight food deprivation of under 200 mg/dl) were excluded from the experiment.

Cell Culture and Differentiation

The L6-GLUT4myc rat myoblast cell line was obtained from Dr. Amira Klip and Dr. Philip Bilan, through a license granted by The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1 × 8; or purchased from Kerafast (ESK202)³⁶. The 3T3-L1-GLUT4myc fibroblast cell line was provided by Dr. Tomoyuki Yuasa, Tokushima University³⁷. L6-GLUT4myc myoblasts were grown in MEMα supplemented with 10% FBS and 1% antibiotics, and then induced to differentiate into myotubes in MEMα supplemented with 2% FBS and 1% antibiotics for 5–8 days. 3T3-L1-GLUT4myc fibroblasts were induced to differentiate into adipocytes as described previously,³⁸ with minor modifications. Briefly, cells were grown to confluence in growth medium: DMEM (Gibco) supplemented with 10% FBS and 1% antibiotics, and then induced to differentiate into adipocytes in growth medium supplemented with 1 μM dexamethasone, 2 μM rosiglitazone, 0.5 mM isobutylmethylxanthine, and 10 μg/mL insulin for two days. The adipocytes were then cultured in growth medium supplemented with 10 μg/mL insulin for a few days before being used for the experiments.

Detection and Quantitation of Cell Surface GLUT4 Using anti-Myc Antibody

Cell surface GLUT4 levels in the L6-GLUT4myc myoblasts and myotubes were determined by antibody binding assay as described previously,^{36,37} with minor modifications. Briefly, cells in a 96- or 24-well plate were starved in serum-free MEMα (0.1% BSA and 1% antibiotics) for 3 h and then treated with the indicated concentrations of DS20060511 and/or insulin for 30 min at 37 °C. After fixation with 4% paraformaldehyde, the cells were incubated with 1% glycine in PBS at 4 °C for 10 min, and blocked with 10% normal goat serum and 3% BSA in PBS at 4 °C for 30 min. The cells were then incubated with anti-c-Myc antibody (sc-40, 1:500) diluted with blocking buffer at 4 °C for 1 h, washed with cold PBS(+), incubated with HRP-conjugated anti-mouse IgG diluted with blocking buffer at 4 °C for 30 min, and then washed again. SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific) was added and the luminescent signal was measured. To investigate GLUT4 translocation in the 3T3-L1-GLUT4myc adipocytes, the cells were prepared in a 24-well plate, and anti-c-Myc antibody (sc-789, 1:1000), HRP-conjugated anti-rabbit IgG and SIGMAFAST OPD reagent (Sigma-Aldrich) were added for optical detection of the cell surface GLUT4myc levels. The GLUT4 translocation activity was normalized to that in the vehicle-treated group.

In vitro Cellular 2-DG Uptake

L6-GLUT4myc myotubes and 3T3-L1-GLUT4myc adipocytes were incubated in the wells of a 24-well plate containing serum-free medium for 3 h at 37 °C and then incubated in glucose-free medium for 30 min at 37 °C. The cells were treated with the indicated concentrations of DS20060511 or insulin for 30 min at 37 °C, followed by the addition of 1 mM 2-deoxy-D-glucose (2-DG) and 0.3 μCi/mL [³H]-2-DG for 10 min. 2-DG uptake was measured with a liquid scintillation counter and normalized to the level in the vehicle-treated group.

Glucose Tolerance Test (GTT)

Mice that had been denied access to food overnight received the indicated oral dose of DS20060511 or vehicle 15 min prior to the glucose load. Then, after the oral glucose load, the blood glucose levels were measured using Glutest Every (Sanwa Kagaku Kenkyusho) in samples obtained from the tail vein at the indicated time points. Plasma insulin concentrations were also measured with an ELISA kit (Morinaga) in blood samples collected from the tail vein at the indicated time points.

Insulin Tolerance Test (ITT)

Mice received the indicated oral dose of DS20060511 or vehicle at the same time as the intraperitoneal injection of insulin at the indicated dose. Blood glucose levels were measured using Glutest Every (Sanwa Kagaku Kenkyusho) in blood samples collected from the tail vein at the indicated time points.

In vivo 2-DG Uptake

Tissue glucose uptake was examined by measuring the uptake of [³H]-2-DG during an intraperitoneal GTT as described previously^{39,40}, with minor modifications. Mice that had been denied access to food overnight received oral administration of 30 mg/kg of DS20060511 or vehicle 15 min prior to the glucose load. The mice then received intraperitoneal glucose administration (1 g/kg containing 100 μCi/kg [³H]-2-DG as tracer), followed by quick removal of the tissues 60 min later. Tissue samples were homogenized in 0.5% perchloric acid and centrifuged, and the supernatants were neutralized with KOH. One aliquot of the supernatants was used for measuring the total radioactivity ([³H]-2-DG and [³H]-2-DG 6-phosphate ([³H]-2-DGP)). A second aliquot of the supernatants was treated with 1N Ba(OH)₂ and 1N ZnSO₄ to remove [³H]-2-DGP, and the [³H]-2-DG count was measured. 2-DG uptake into the tissue, which is rapidly metabolized to 2-DGP in the tissue, was estimated by subtracting the count of [³H]-2-DG from the total count.

2-DG Uptake in Isolated Skeletal Muscle

Soleus or EDL muscle was removed from mice that had been denied access to food overnight under isoflurane anesthesia and incubated with oxygenated (95% O₂/5% CO₂) Krebs-Henseleit Bicarbonate (KHB) buffer (118.1 mM NaCl, 4.7 mM KCl, 1.1 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, pH7.4) in the presence of 11.1 mM glucose. Muscles were then treated with the indicated concentrations of compounds in KHB buffer containing 11.1 mM glucose and 8 mM mannitol for 10 min. Thereafter, the muscles were rinsed with KHB buffer containing 8 mM mannitol and the compounds for 5 min. Lastly, the muscles were treated with compounds in KHB buffer containing 1 mM 2-DG (with 0.3 μCi/mL [³H]-2-DG) and 8 mM mannitol (with 0.03 μCi/mL [¹⁴C]-mannitol) for 10 min. The ¹⁴C and ³H specific activities were counted with a liquid scintillation counter. The specific uptake of 2-DG was calculated by subtracting the non-specific uptake of mannitol from the total 2-DG uptake. To investigate the effects of muscle contraction, muscle contraction was induced by electrical stimulation with 5 Hz (1 ms pulse duration, 100 V) for 10 min (SEN-5201, Nihonkoden).

Plasma Membrane Fractionation of the Skeletal Muscle

Mice that had been denied access to food overnight received DS20060511 (10 mg/body), insulin (5 unit/body) or saline (vehicle control) via the inferior vena cava under isoflurane anesthesia, and 10 min later, the hindlimb muscles were excised. The plasma membrane fraction of the skeletal muscle was prepared as described previously^{41,42}. Briefly, the hindlimb muscles were homogenized in Buffer A (20 mM HEPES, 1 mM EDTA, 1 mM PMSF and protease inhibitor) containing 250 mM sucrose. The muscle homogenates were centrifuged at $2,000 \times g$ for 10 min to remove any unhomogenized muscle fibers. The supernatants were then centrifuged at $19,000 \times g$ for 20 min. The pellets were resuspended in 3 mL Buffer A, layered on a 6 mL sucrose cushion (38% sucrose in Buffer A) and centrifuged at $100,000 \times g$ for 60 min. The membrane fraction recovered on top of the sucrose cushion was resuspended in Buffer A and centrifuged at $40,000 \times g$ for 20 min. The pellet was designated as the plasma membrane fraction and subjected to immunoblotting.

Sample preparation for immunoassays of insulin- and AMPK-signaling molecules

For the analyses of insulin and AMPK signal transduction, mice that had been denied access to food overnight received DS20060511 (10 mg/body), insulin (5 unit/body) or saline (vehicle control) via the inferior vena cava under isoflurane anesthesia and 10 min later, the hindlimb muscles were excised for Western blot analysis. Tissue samples were lysed and the lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatants were collected and the protein concentrations were determined by BCA assay. Immunoprecipitation of IR β and IRS1 was performed as described previously¹⁰, using specific antibodies against IR β (Santa Cruz, sc-711), IRS1 (Millipore, 06-248) and for detection of phosphotyrosine (Millipore, 05-321). Five milligrams the extracts were incubated with specific antibodies against IRS1 for 1 h at 4 °C. Then, protein G-Sepharose was added, followed by incubation for 2 h at 4 °C. After washing three times, the immunocomplexes were subjected to immunoblotting.

Immunoblotting

Phosphorylated or total protein of IR β and IRS1 was resolved on 7% SDS PAGE and analyzed using specific antibodies against IR β , IRS1, and phosphotyrosine. Analyses were also conducted for phosphorylated Akt (1:2000), Akt (1:2,000), AS160 (1:1,000), phospho-AS160 (1:1,000), AMPK α (1:1,000), phospho-AMPK α (1:1,000), GLUT4 (1:200), Na,K-ATPase α (1:1,000), GAPDH (1:1,000) by immunoblotting with specific antibodies after the tissue lysates were resolved on SDS-PAGE and transferred to a PVDF membrane using Trans-Blot Turbo transfer system (Bio-Rad Laboratories). Bound antibodies were detected with HRP-conjugated secondary antibodies using ECL detection reagents (Amersham Biosciences).

Fluorescence Immunostaining

Differentiated L6-GLUT4myc myotubes were prepared in a collagen-coated 4-well chamber slide. After serum starvation for 3 h, the cells were stimulated with insulin or DS20060511 for 15 min at 37°C. Cells

were rinsed with cold PBS(+), fixed with 4% paraformaldehyde for 30 min on ice and blocked with 1% BSA and 10% normal goat serum in PBS(+) for 30 min at room temperature. Surface GLUT4myc staining was carried out without cell membrane permeabilization. Cells were incubated with anti-c-Myc antibody for 30 min at room temperature, followed by treatment with 0.1% Triton-X. After blocking, the cells were incubated with Alexa 488-conjugated secondary antibody and Alexa 594-conjugated phalloidin for 30 min at room temperature. Fluorescence images were obtained with a Leica TSC-SP8 confocal microscope. Specimens were scanned along the z-axis and a single composite image was generated by the maximal projection method using the LAS software (Leica Microsystems).

Indirect calorimetry under treadmill running

The mice received oral administration of 30 mg/kg of DS20060511 or vehicle 15 min before they were made to run. At 0 min, the treadmill was started at a velocity of 10 m/min, with the speed increased by 2 m/min every 3 min. The respiratory quotient, and glucose and fat oxidation under treadmill running were analyzed by ARCO-2000 (ARCO system Inc.).

Repeated DS20060511 treatment of the db/db mice

db/db mice were acclimatized to a 6-h restricted feeding pattern from 10 am to 4 pm prior to the start of the experiment. For 28 days from 6 weeks of age, the mice received oral administration of 10 mg/kg of DS20060511 or vehicle once a day, 15 min prior to their feeding. On day 1 and day 28, the blood glucose levels were measured between 10 am to 4 pm. Daily food intake was monitored throughout the study, and the body weight, blood glucose and plasma insulin were measured on day 1 and day 28. The hemoglobin A1c (HbA1c) level was also measured with a HLC-723G8 Automated Glycohemoglobin Analyzer on day 1 and day 28 (Tosoh India Pvt. Ltd.).

Antibodies

Antibodies against c-Myc (9E10, sc-40 and A-14, sc-789), Glut4 (C-20, sc-1608) and IR β (C-19, sc-711) were purchased from Santa Cruz Biotechnology. Antibodies against Akt (9272), phospho-Akt (9271), AMPK α (2532), phospho-AMPK α (2531), Na,K-ATPase α (3010) and GAPDH (2118), and anti-mouse IgG (7076) and anti-rabbit IgG (7074) were purchased from Cell Signaling Technology. Rabbit polyclonal antibody against IRS1 (06-248), mouse monoclonal antibody against phosphotyrosine (05-321), rabbit antiserum against AS160 (07-741), and rabbit polyclonal antibody against phospho-AS160 (07-802) were purchased from Millipore. HRP-conjugated donkey anti-goat IgG (V8051) was purchased from Promega. HRP-conjugated goat anti-rabbit IgG (111-035-144) and goat anti-mouse IgG (115-035-003) were purchased from Jackson ImmunoResearch.

Statistical Analysis

Values are expressed as the means \pm SEM. Differences between two groups were assessed using Student's t-tests. Statistical differences among multiple groups were evaluated by ANOVA followed by indicated post-hoc multiple comparison.

Declarations

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Author contributions

SF, JT, MK, TeK, NK, and TK conceived and designed the experiments; SF, JT, and AN performed the experiments; SF and TJ analyzed the data; JT, TeK, and HK wrote the manuscript; TeK, NK, and TK reviewed and revised the manuscript.

Competing interest

SF, JT, MK, AN, and HK are employees of Daiichi Sankyo Co., Ltd. (Tokyo, Japan).

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Figures

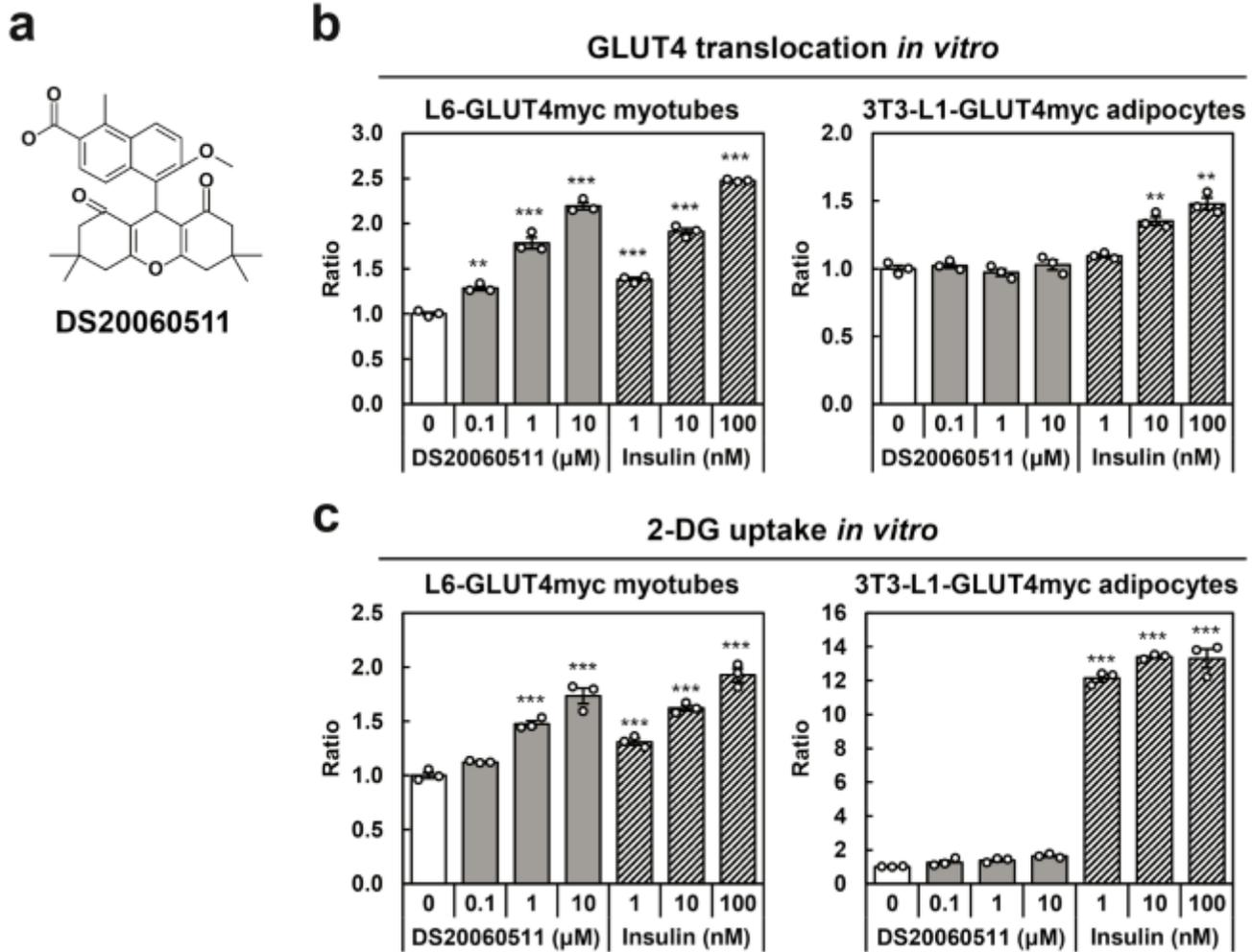
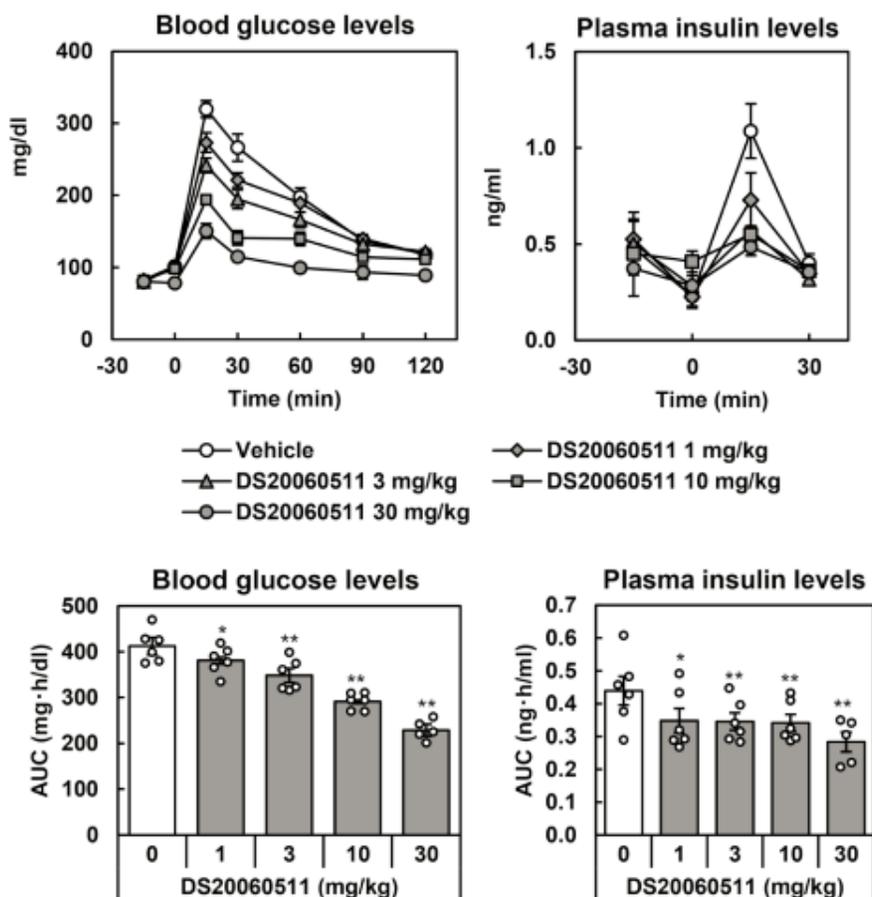
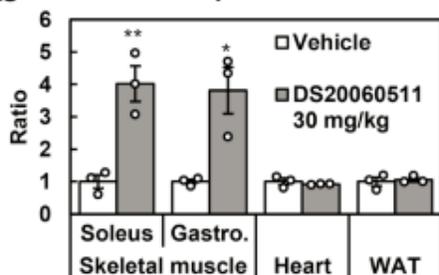
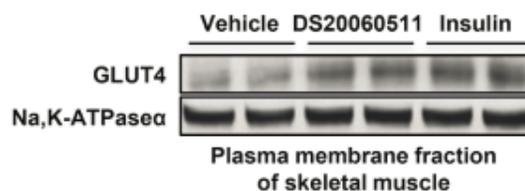


Figure 1

A xanthene derivative DS20060511 induced skeletal muscle-specific GLUT4 translocation. a Chemical structure of DS20060511. b Concentration-dependent induction of GLUT4 translocation by DS20060511 and insulin in L6-GLUT4myc myotubes (left panel) and 3T3-L1-GLUT4myc adipocytes (right panel). c 2-DG uptake evaluated in L6-GLUT4myc myotubes (left panel) and 3T3-L1-GLUT4myc adipocytes (right panel). Values are the means \pm SEM, $n = 3$. ** $P < 0.01$, *** $P < 0.001$ vs. control by one-way ANOVA, followed by Dunnett's test.

a**Glucose tolerance test****b****2-DG uptake *in vivo*****c****GLUT4 translocation *in vivo*****Figure 2**

Treatment with DS20060511 decreases the blood glucose levels via inducing GLUT4 translocation and increasing skeletal muscle glucose uptake. a Blood glucose and plasma insulin levels during oral GTT in the C57BL/6 mice ($n = 5-6$). The mice received oral administration of vehicle or DS20060511 at the indicated dose, 15 min prior to glucose administration (1.5 g/kg). Values are the means \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs. vehicle by one-way ANOVA, followed by Williams' test. b [3H]-2-DG uptake in the soleus

muscle, gastrocnemius muscle (Gastro.), heart, and white adipose tissue (WAT) at 60 min during the intraperitoneal GTT in the C57BL/6 mice (n = 3). The mice received oral administration of vehicle or DS20060511 (30 mg/kg), 15 min prior to glucose administration (1 g/kg glucose containing [3H]-2-DG). Values are the means \pm SEM. *P < 0.05, **P < 0.01 vs. vehicle. c Protein levels of GLUT4 and Na,K-ATPase in the plasma membrane fraction of the hindlimb muscles excised from the C57BL/6 mice (n = 2) treated with DS20060511 (10 mg/body), insulin (5 unit/body), or saline as vehicle via the inferior vena cava 10 min after the treatment.

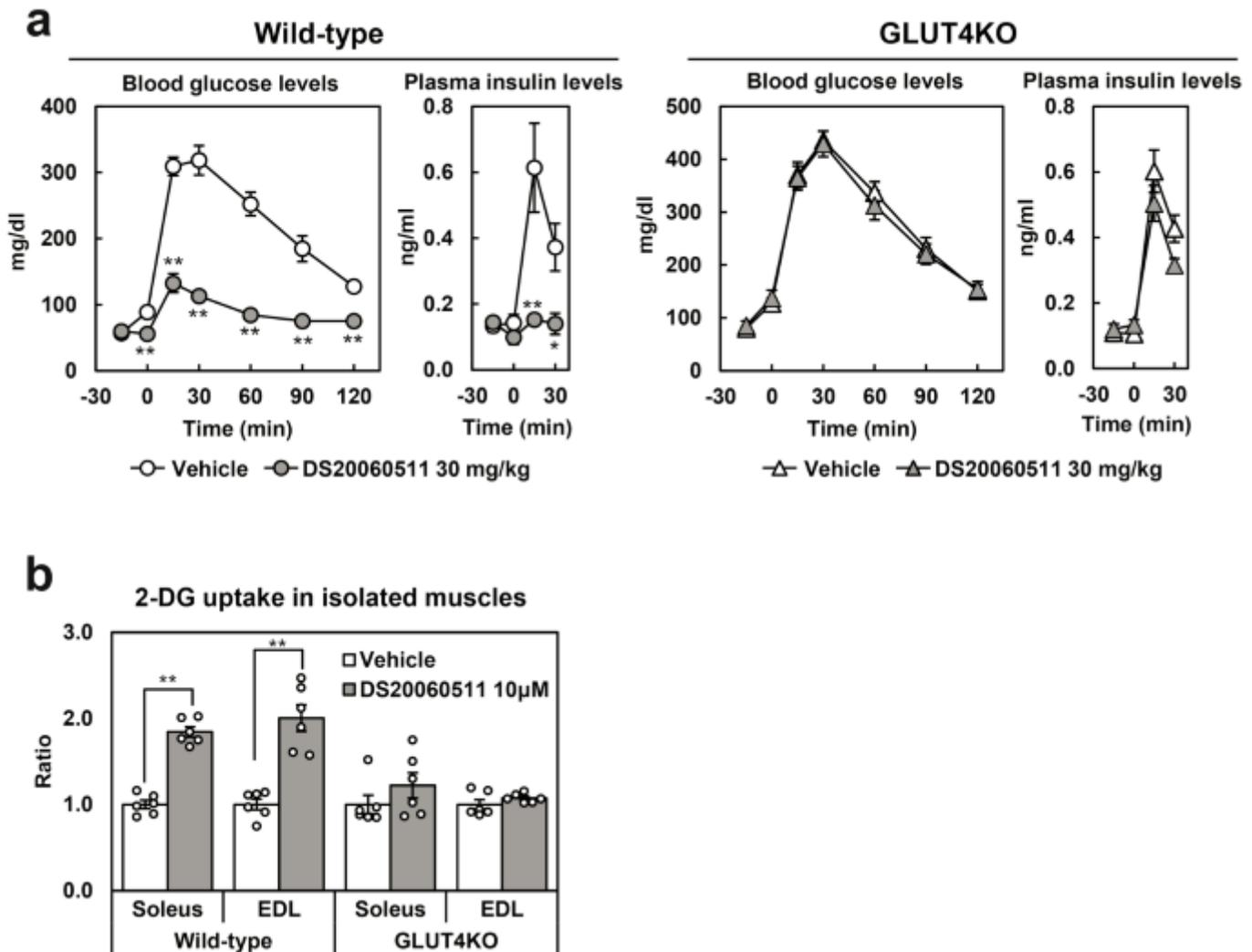


Figure 3

The glucose-lowering effect of DS20060511 is totally dependent on GLUT4. a Blood glucose and plasma insulin levels during oral GTT in wild-type (WT, n = 5) and GLUT4 knockout (KO, n = 6) mice. Mice received oral administration of vehicle or DS20060511 (30 mg/kg), 15 min prior to the glucose administration (1.5 g/kg). b DS20060511-stimulated [3H]-2-DG uptake in the isolated soleus and EDL muscles excised from the WT (n = 5) and KO (n = 6) mice. Values are the means \pm SEM. *P < 0.05, **P < 0.01 vs. vehicle.

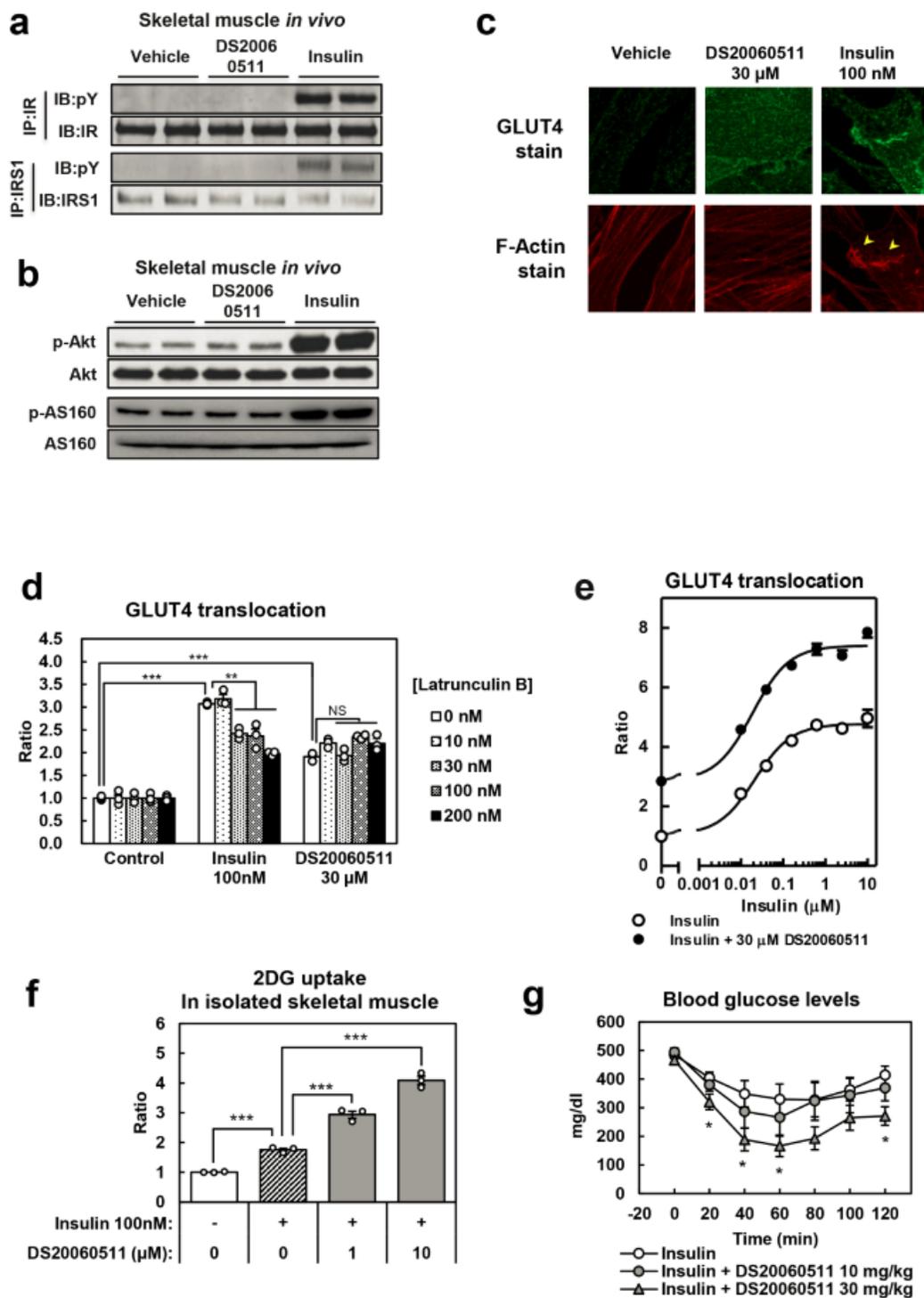


Figure 4

The mechanism underlying DS20060511-induced GLUT4 translocation is distinct from that of insulin-induced GLUT4 translocation. a, b Phosphorylation of IR β , IRS1, Akt (Ser473) and AS160 of the hindlimb muscles excised from C57BL/6 mice (n = 2) treated with DS20060511 (10 mg/body), insulin (5 unit/body), or saline as vehicle via inferior vena cava 10 min after the treatment. c Fluorescence immunostaining of cell surface GLUT4 and intracellular actin fibers in L6-GLUT4myc myotubes treated

with 30 μ M of DS20060511 or 100 nM of insulin. Arrowheads indicate the characteristic ruffled structure of the polymerized actin (lower panel) and actin-associated surface GLUT4 (upper panel). d GLUT4 translocation activity of 30 μ M DS20060511 or 100 nM insulin in the presence of the actin polymerization inhibitor, Latrunculin B, at the indicated concentrations. Values are the means \pm SEM. **P < 0.01 vs. 0 nM Latrunculin B by one-way ANOVA followed by Williams' test. ***P < 0.001 vs. control. e Concentration dependent insulin-stimulated GLUT4 translocation activity in L6-GLUT4myc myotubes with or without 30 μ M DS20060511. f Concentration dependent DS20060511-stimulated 2-DG uptake with or without 100 nM insulin in isolated muscles from C57BL/6 mice (lower panel; n = 3). Values are the means \pm SEM. ***P < 0.001. g Blood glucose levels during insulin tolerance test in STZ-treated C57BL/6 mice (n = 6–7). Vehicle or indicated dose of DS20060511 was given orally at the same time as 0.1 U/kg insulin injection intraperitoneally. Values are the means \pm SEM. *P < 0.05, vs. vehicle.

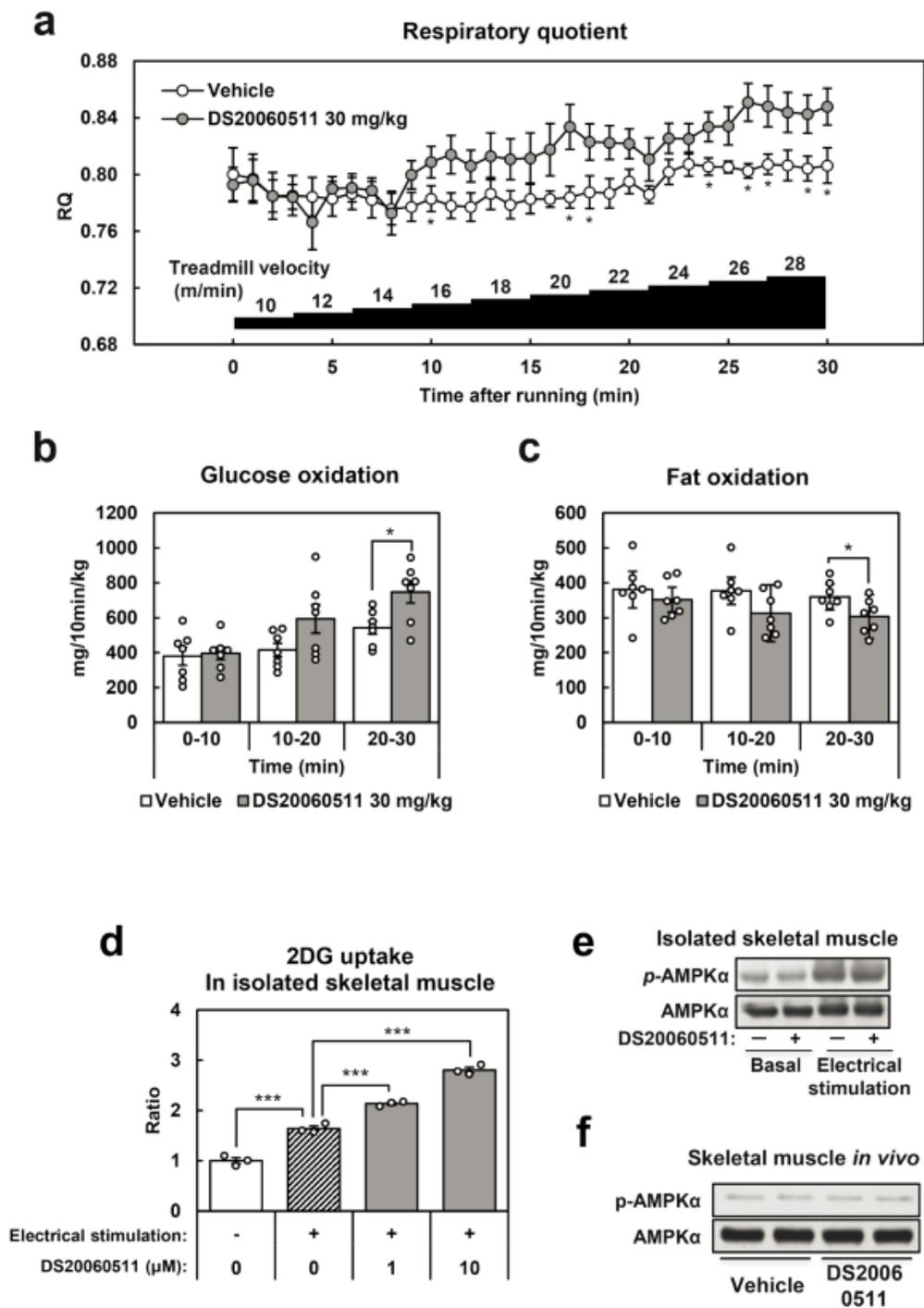


Figure 5

DS20060511 increases glucose oxidation during exercise. a-c Respiratory quotient, glucose oxidation, and fat oxidation during step-wise treadmill running in C57BL/6 mice ($n = 7$). Vehicle or DS20060511 (30 mg/kg) was given orally 15 min before starting running. Treadmill started from the velocity of 10 m/min and increased by 2 m/min each 3 min. Values are the means \pm SEM. * $P < 0.05$ vs. the vehicle. d Concentration dependent DS20060511-stimulated 2-DG uptake with or without muscle contraction (5 Hz

electrical stimulation) in isolated muscles from C57BL/6 mice (n = 3). *P < 0.05 vs. no electrical stimulation with each concentration of DS20060511. e Muscle contraction (5 Hz electrical stimulation) induced AMPK (Thr172) phosphorylation with or without 10 μ M DS20060511 in isolated muscles from C57BL/6 mice (n = 3). f Phosphorylation levels of AMPK α of the hind limb muscles excised from C57BL/6 mice (n = 3) treated with DS20060511 (10 mg/body) or saline as vehicle via inferior vena cava 10 min after the treatment.

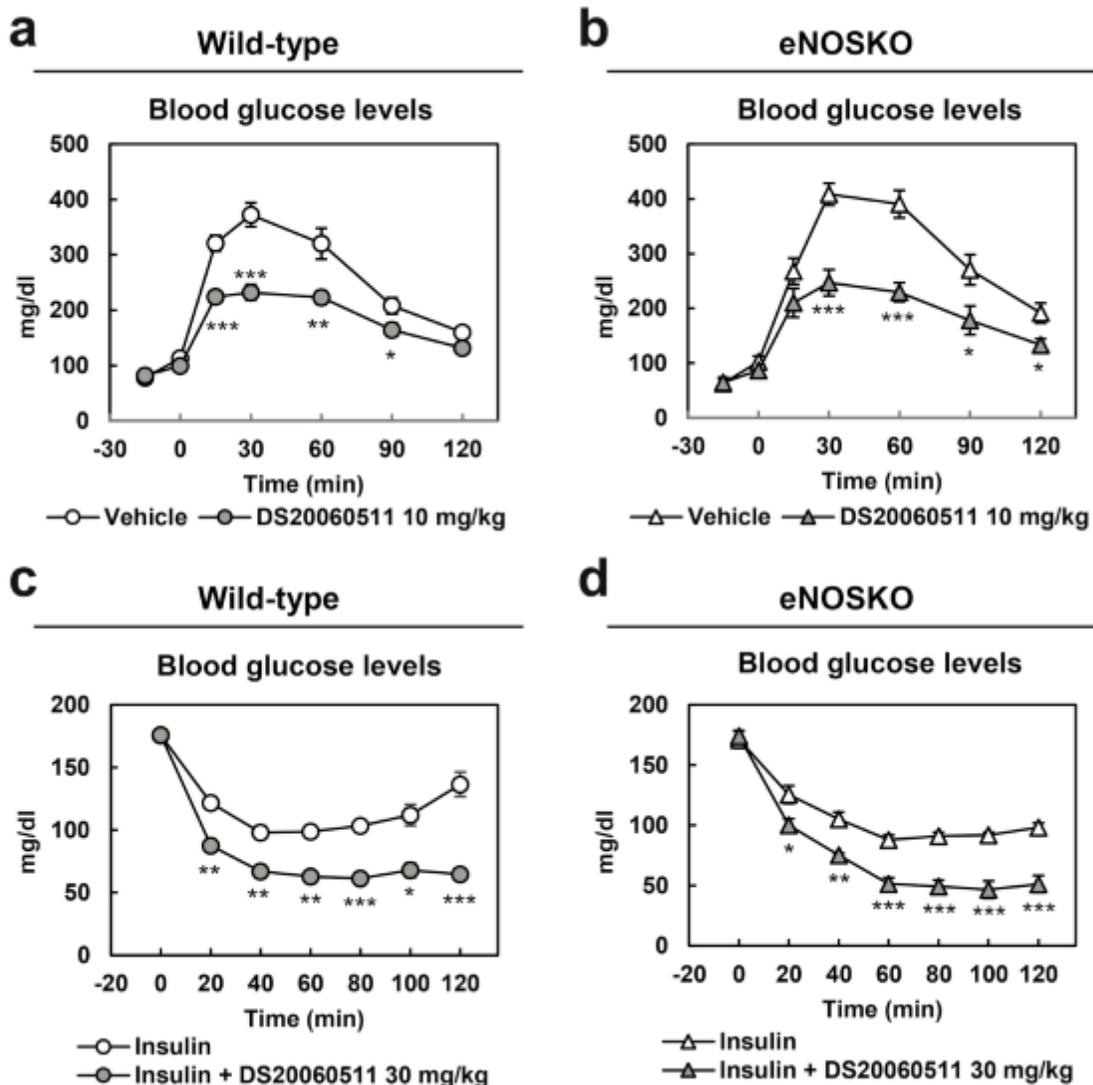


Figure 6

DS20060511 treatment decreases blood glucose levels in both WT and eNOSKO mice during oral GTT and ITT a, b Blood glucose levels during oral GTT in wild-type (WT, n = 5) and eNOS knockout (KO, n = 5–6) mice. Mice received vehicle or DS20060511 (10 mg/kg) orally 15 min before the glucose administration (3.0 g/kg). c, d Blood glucose levels during ITT in WT (n = 4) and KO (n = 5) mice. Vehicle or indicated dose of DS20060511 was given orally at the same time as 0.5 U/kg insulin injection intraperitoneally. Values are the means \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.

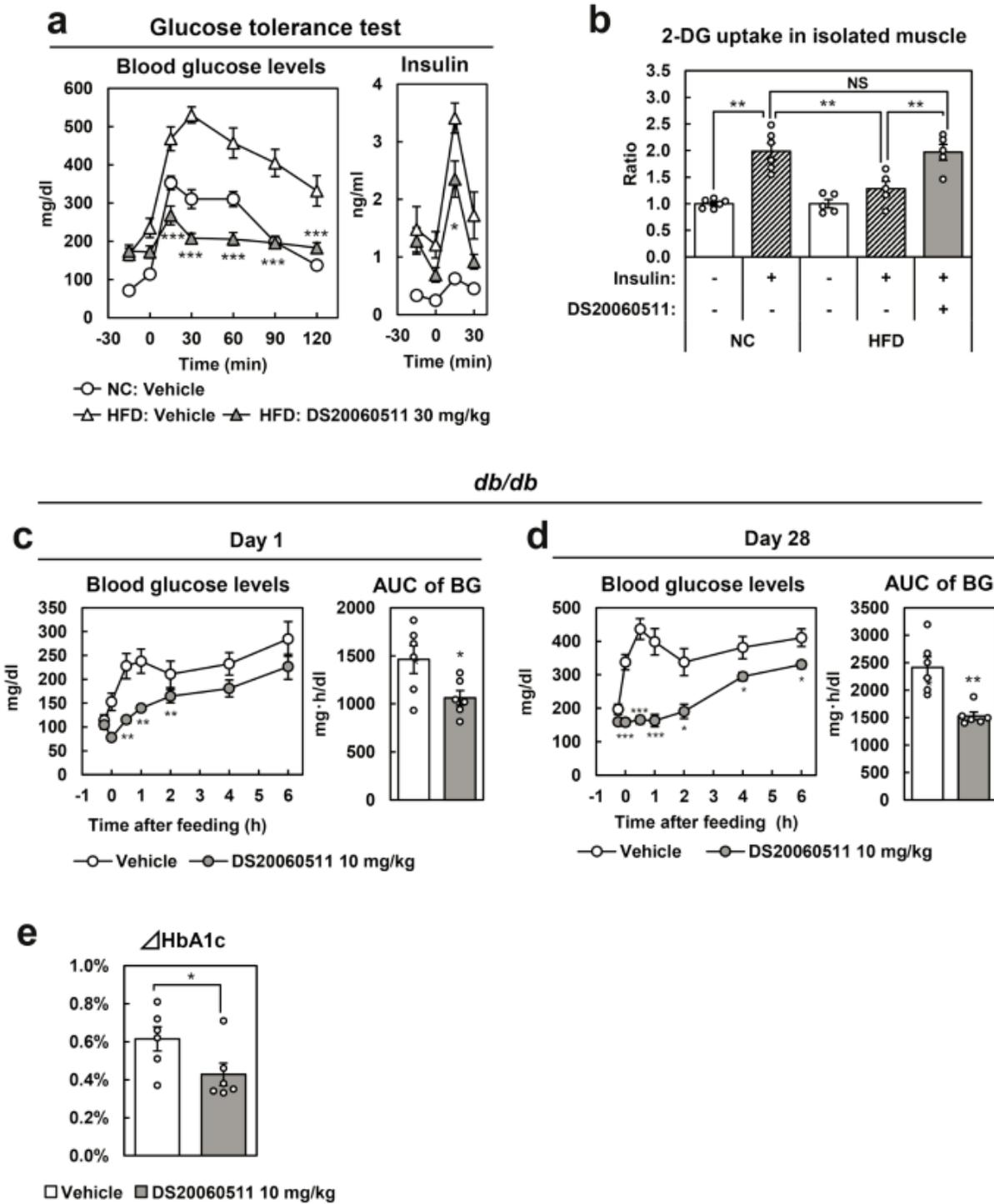


Figure 7

Acute and chronic DS20060511 treatment improves glucose intolerance in HFD-fed and db/db mice. a Blood glucose and plasma insulin levels during oral GTT in normal chow (NC)- and HFD-fed mice (n = 5). Vehicle or DS20060511 (30 mg/kg) was given orally 15 min before the oral glucose administration (1.5 g/kg). Values are the means ± SEM. *P < 0.05, **P < 0.01 vs. the HFD vehicle. b Effects of 10 μM DS20060511 and 100 nM insulin on 2-DG uptake in muscles isolated from normal chow-fed (n = 6) and

HFD-fed (n = 5) mice. NC, normal chow. Values are the means \pm SEM. **P < 0.01 vs. the vehicle by one-way ANOVA followed by Tukey's test. c, d Changes in blood glucose levels and AUC during re-feeding at day 1 and day 28 in db/db mice treated with DS20060511 chronically. e Change of HbA1c levels of db/db mice in 4 weeks. Values are the means \pm SEM. *P < 0.05, **P < 0.01 vs. vehicle.

Supplementary Files

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