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Research Article

NPC43 Activates Insulin Receptor in White Adipose Tissue and Stimulates Adipocyte Glucose Uptake to Mitigate Hyperglycemia in Type 1 And 2 Diabetic Mice

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Abstract

Finding an affordable and orally effective small compound, which can replace insulin to activate insulin receptor (INSR) in insulin-sensitive organs such as white adipose tissue (WAT), represents an important advancement in the management of hyperglycemia in diabetes. Adenosine, 5'-Se-methyl-5'-seleno-, 2',3'-diacetate (NPC43) is a recently identified non-peptidyl, small compound that can target liver and skeletal muscle to activate Insr in type 1 diabetic (T1D) and type 2 diabetic (T2D) mice (Lan et al, Cell Mol Life Sci, 2020 and BMJ Open Diabetes Res Care, 2020). However, whether NPC43 can target WAT to activate adipose Insr signaling for glucose uptake to mitigate hyperglycemia in diabetes remains unclear. In this study, we found that intraperitoneal administration of NPC43 into T2D *Lepr*$^{db/db}$ mice caused a significant decrease in blood glucose levels and a marked increase in Insr and AS160 phosphorylation in WAT. A significant increase in Insr and AS160 phosphorylation were also observed in WAT of *Lepr*$^{db/db}$ and streptozotocin-induced T1D mice following oral NPC43 treatment. In addition, NPC43 treatment was able to directly activate Insr/AS160 signaling and promote glucose uptake in differentiated NIH3T3L1-MBX adipocytes. Furthermore, a cooperative action between NPC43 and insulin in stimulating glucose uptake was observed in differentiated NIH3T3L1-MBX adipocytes. Together, these results indicate that NPC43 can target WAT to activate adipose Insr/AS160 signaling for glucose uptake to mitigate hyperglycemia in T1D and T2D. This, coupled with our earlier findings that NPC43 is effective when administered orally, underscores the potential of this compound as an effective, affordable, non-injectable alternative to insulin.

Keywords: Insulin mimetic; Insulin receptor; Type 1 and 2 diabetic mouse; White adipose tissue; Differentiated adipocytes; Glucose uptake

Introduction

Adipose tissue is an insulin-sensitive organ that plays a role in glucose homeostasis [1-4]. Increases in post-prandial blood glucose levels stimulate the secretion of insulin from pancreatic beta cells. Insulin binding to the extracellular domain of insulin receptor (Insr) activates intracellular Insr tyrosine kinase (manifested by auto-phosphorylation of tyrosine residues at 1146, 1150 and 1151 of Insrβ) [5,6]. Subsequently, protein kinase-B (Akt) is activated to stimulate the phosphorylation of an Akt downstream target, AS160 (also known as TBC1D1/4) [7-9]. Phosphorylation of AS160 facilitates the translocation of cytosolic transporter protein Glut4 vesicles to the plasma membrane and promotes glucose transport into adipose tissues and other insulin target organs, such as skeletal

muscle and liver tissues, thereby reestablishing normoglycemia [1,9-11]. It has been reported that adipose tissue is responsible for about 10% of whole-body glucose uptake [12,13]. Impaired insulin-stimulated glucose uptake in adipose tissue is associated with insulin resistance and type 2 diabetes (T2D) [4,12,14-19]. In type 1 diabetes (T1D), due to the failure of insulin production by the pancreas [20], glucose uptake in adipose tissue is completely blunted, which contributes to hyperglycemia. There is an urgent need for affordable alternatives to insulin for the treatment of both T1D and T2D, since the cost of insulin is becoming unaffordable, even in developed countries [21-23], and the number of diabetic patients requiring insulin is rapidly increasing [24]. Therefore, finding an orally effective small molecule, which can activate INSR in white adipose tissue (WAT) in an insulin-independent manner, may represent an important advancement in the treatment of hyperglycemia in both T1D and T2D.

Adenosine, 5'-Se-methyl-5'-seleno-, 2',3'-diacetate (NPC43) is a recently identified, non- peptidyl, small compound which not only can replace insulin to attenuate hyperglycemia in streptozotocin (STZ)-induced T1D mice but also can effectively mitigate hyperglycemia and improve insulin resistance in hyperinsulinemic T2D *Lepr*$^{db/db}$ mice [25,26]. In the insulin-resistant *Lepr*$^{db/db}$ mice, intraperitoneal (i.p.) administration of NPC43 restores normal Insr/Akt/AS160 signaling in both skeletal muscle and liver tissues, and Insr/Akt/Foxo1 signaling in the liver to inhibit the expression of *G6pc* for gluconeogenesis [25]. In insulin-deficient, STZ-induced T1D mice, oral NPC43 treatment can also activate Insr/Akt/AS160 signaling in both skeletal muscle and liver tissues [26]. Additionally, in cultured liver and skeletal muscle cells, NPC43 mimics insulin to directly activate INSR/AKT/AS160 signaling for promoting hepatic and skeletal muscle glucose uptake, and INSR/AKT/FOXO1 signaling for inhibiting glucose production in human liver cells [25]. NPC43 also cooperates with insulin to stimulate skeletal muscle glucose uptake and attenuates hepatic *G6pc*-driven gluconeogenesis [25]. Furthermore, NPC43 can directly interact with Insrα [25,27] and activates native liver Insr protein in a cell-free, in vitro phosphorylation system [25]. Thus, liver and gastrocnemius are two identified target organs of NPC43 in which NPC43 can restore or activate Insr signaling to counter hyperglycemia in T1D and T2D mice [25,26].

To date, whether or not NPC43 targets WAT to activate adipose Insr/AS160 signaling (key for glucose uptake) for mitigation of hyperglycemia in both types of diabetes remains unknown. To address this, the activities of NPC43 in attenuating hyperglycemia and activating Insr/AS160 signaling in WAT were investigated in T2D *Lepr*$^{db/db}$ mice following i.p. and/or oral administration. In addition, the ability of NPC43, as an oral insulin-replacing agent, to activate Insr/AS160 signaling in WAT was also examined in STZ-induced T1D mice. Last but not least, the direct effects of NPC43 on the activation of Insr/AS160 signaling and glucose uptake were investigated in differentiated NIH3T3L1-MBX adipocytes.

Materials and Methods

Chemical reagents

NPC43 was synthesized as described [25] and the purity of NPC43 was verified to be ≥ 99%, as determined by mass spectrometry analysis using a UPLC-ESI-VionIMS-QTOF instrument (Waters Corp., Milford, MA) [25]. Insulin (Cat #I9278), STZ (Cat #S0130), isobutylxanthine (Cat #5879), dexamethasone (Cat #D4902) and rosiglitazone (Cat #R2408) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

STZ-induced T1D male mice were generated as previously described [26,28]. Male T2D *Lepr*$^{db/db}$ mice (C57BL/6J strain, five to eight-week-old) were purchased from The Jackson Laboratory (Bar Harbor, Maine). These mice were housed in the pathogen-free vivarium with free access to chow and water. Room temperature at the vivarium was kept at approximately 25°C, with a relative humidity of 60% and a 12/12 hour (hr) light/dark cycle. For tissue collection, all animals were anesthetized with ketamine [87.5 milligrams per kilogram body weight (mpk)]/xylazine (12.5 mpk) by i.p. injection to avoid animal suffering. All animal studies were pre-approved by Alltech, Inc., and performed according to the U.S. National Institute of Health's Animal Welfare guidelines.

Analysis of blood glucose levels, body weight and collection of epididymal WAT from *Lepr*$^{db/db}$ mice following i.p. NPC43 treatment

Administration of NPC43 to *Lepr*$^{db/db}$ mice by i.p. injection was described previously [25]. In brief, male *Lepr*$^{db/db}$ mice at 35 days of age were injected daily with physiological saline containing 0.2% (v/v) dimethyl sulfoxide (DMSO) or NPC43 (0.136 mpk). At day 30 post-i.p. treatments, animals were fasted for 4 hr and then subjected to blood glucose analysis. At day 58 and 86 post-i.p. treatments, animals were fasted overnight and subjected to blood glucose analysis as described [25]. Mouse body weights before and after i.p. treatment for 86 days were recorded using an electronic weigh balance. Epididymal WAT from these mice after i.p. treatment for 86 days was collected and stored at -80°C.

Collection of epididymal WAT from *Lepr*$^{db/db}$ mice after oral NPC43 treatment

Oral administration of NPC43 in *Lepr*$^{db/db}$ mice was described previously [25]. In brief, male *Lepr*$^{db/db}$ mice (65-day-old) were fed daily (by oral gavage) with 0.5% (w/v) carboxymethylcellulose (CMC) or NPC43 (5.4 mpk). At 9 days post-oral gavage, *Lepr*$^{db/db}$ mice were fasted for 2 hr and epididymal WAT from these mice was collected and stored at -80°C.

Collection of epididymal WAT from STZ-induced T1D mice after oral NPC43 treatment

STZ-induced T1D mice were orally administered with NPC43 as previously described [26]. In brief, male STZ-induced T1D mice at 48 days post-STZ treatment were fasted for 2 hr and then subjected to oral gavage with 5.4 mpk NPC43 or its vehicle [1% (v/v) DMSO/physiological saline, referred to saline] for 5 minutes. Epididymal WAT from these saline- or NPC43-treated T1D mice were collected and stored at -80°C.

Differentiation of NIH3T3L1-MBX fibroblasts into mature adipocytes

Mouse NIH3T3L1-MBX fibroblast cells were purchased from ATCC (Manassas, Virginia). These fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Differentiation of NIH3T3L1-MBX fibroblasts into mature adipocytes was performed according to the Promega's Technical Manual of Glucose Uptake-Glo™ assay (Cat #J1343, Madison, WI. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/tm467-glucose-uptakeglo-assay.pdf?sc_lang=en?la=en). Images of cultured NIH3T3L1-MBX fibroblast cells (at day 3 of culture) and mature adipocytes at day 22 of culture [also referred to post-differentiation day (PD) 8] were captured under a Zeiss-inverted phase-contrast microscope.

Protein preparation of mouse WAT and cultured adipocytes after NPC43 treatment

Frozen epididymal WAT from the above vehicle- and NPC43-treated *Lepr*$^{db/db}$ or STZ-induced T1D mice were processed for protein isolation using a RIPA buffer containing complete proteinase and phosphatase inhibitors (ThermoFisher Scientific, Waltham, MA), as described previously [29,30]. For isolation of protein samples from NIH3T3L1-MBX adipocytes, mature cells at PD8 were serum-starved overnight and then incubated with 0.06% DMSO or 3.8 μM NPC43 for 3 minutes. Protein samples of NIH3T3L1-MBX adipocytes, following DMSO or NPC43 treatments, were isolated using a RIPA buffer containing complete proteinase and phosphatase inhibitors as described previously [25]. Protein concentrations in these samples isolated from mouse WAT or NIH3T3L1-MBX adipocytes were determined using a Micro-BCA protein assay kit (Thermo Scientific-Piece Biotechnology, Rockford, IL).

Western blot analysis

Protein samples isolated from epididymal WAT (8-10 μg protein /mouse) or NIH3T3L1-MBX adipocytes (7.5 μg protein/sample) were subjected to Western blot analysis, as described previously [25]. Primary antibodies against phosphorylated-Insrβ (pInsrβ) at Y1146 (Cat #3021) or at Y1150 and 1151(Cat #3024), Insrβ (Cat #3025), AS160 (Cat #2670), Gapdh (Cat #5174) and β-tubulin (Cat #2146) were purchased from Cell Signaling Technology (Danvers, MA), while primary antibodies against phosphorylated-AS160 (pAS160) at T642 (Cat #44-1071G) were from ThermoFisher Scientific (Waltham, MA). Protein band densities in the Western blots were determined using the NIH ImageJ software [31] to obtain the ratio of phosphorylated form to total protein level of Insrβ and AS160.

Glucose uptake assay in NIH3T3L1-MBX adipocyte

Glucose uptake assays in NIH3T3L1-MBX adipocytes were performed according to Promega's Technical Manual of Glucose Uptake-Glo™ assay (Cat #J1343, Madison, WI). In brief, mature NIH3T3L1-MBX adipocytes at PD7 were pretreated with 0.06% DMSO (NPC43 solvent) or NPC43 (0.95, 1.9, 3.8 and 7.6 μM) in serum-free DMEM media overnight. At PD8, those pretreated adipocytes were incubated with 0.06% DMSO (basal control), insulin (0.1 and 1 nM), NPC43 (0.95, 1.9, 3.8 and 7.6 μM), or both insulin (0.1 nM) and NPC43 (0.95, 1.9 and 3.8 μM) in serum- and glucose-free DMEM media at 37°C for 1 hr. Following these treatments, NIH3T3L1-MBX adipocytes were incubated with 1 μM 2-deoxyglucose (2DG) at room temperature for 10 minutes. These 2DG-treated cells were then subjected to glucose uptake analysis using Promega's Glucose Uptake-Glo Assay kit (Cat #J1343, Madison, WI), according to the manufacturer's protocol. Luminescence intensities (RLU) in the sample were determined using a 0.5-second integration on the Bio-Tek luminometer.

Statistical analysis

Quantitative data are presented as the mean ± SEM (standard error). All bar graphs were generated using the GraphPad Prism 9 software. A *Student's t-test* was performed to determine the statistical significance of difference between the control and treatment groups with a P value less than 0.05 being deemed significant.

Results

Decreased blood glucose levels and activated adipose Insr/AS160 signaling, without effects on body weight in *Lepr*$^{db/db}$ mice after i.p. NPC43 treatment

As shown in Figure 1a, blood glucose levels in *Lepr*$^{db/db}$ mice following short-term fasting (i.e., 4 hr) were significantly decreased after i.p. NPC43 treatment for 30 days. A more pronounced 31% and 45% decrease in blood glucose levels was observed in overnight-fasted *Lepr*$^{db/db}$ mice after i.p. NPC43 treatment for 58 and 86 days, respectively (Figure 1a). These results confirmed that i.p. NPC43 treatment can attenuate hyperglycemia in *Lepr*$^{db/db}$ mice, as observed previously [25]. We also performed body-weight analysis of *Lepr*$^{db/db}$ mice before and after i.p. treatment for 86 days. As shown in Figure 1b, there was no significant difference in body weight between saline-and NPC43-treated *Lepr*$^{db/db}$ mice before or after i.p. treatment.

Western blot analysis showed that there was a visible increase in protein expression of pInsrβ at Y1150/1151 (a marker of activated Insr [6]) and pAS160 at T642, but not total Insrβ or AS160 protein, in epididymal WAT of *Lepr*$^{db/db}$ mice after i.p. NPC43 treatment for 86 days (*vs.* saline-treated *Lepr*$^{db/db}$ mice) (Figure 1c). Quantitative analysis showed that there was a significant 3.73- and 3.95-fold increase in the ratio of pInsrβ and pAS160 protein levels to total Insrβ and AS160 protein levels, respectively, in the WAT of *Lepr*$^{db/db}$ mice after i.p. NPC43 treatment (Figure 1d). These results demonstrated that i.p. NPC43 treatment activated Insr/AS160 signaling in WAT of *Lepr*$^{db/db}$ mice.

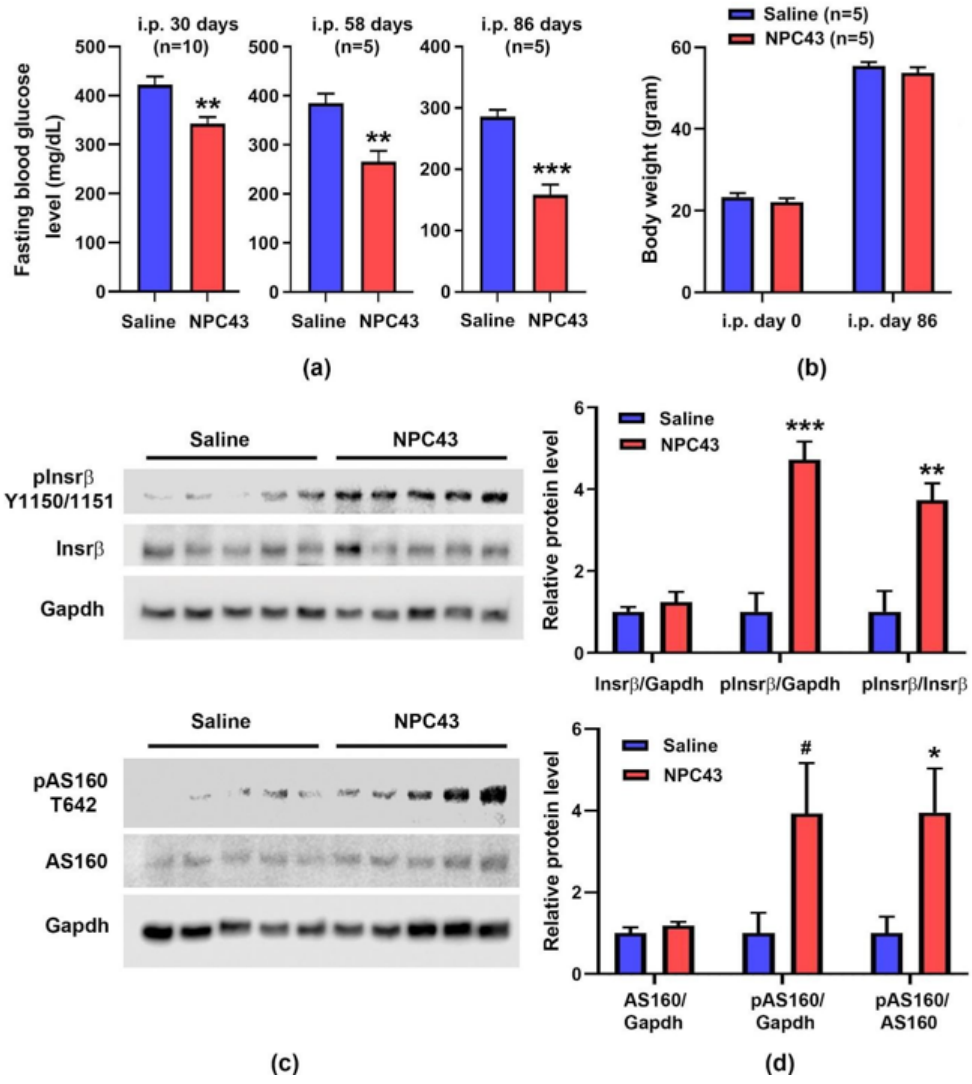

Figure 1. Decreased blood glucose levels and activated adipose Insr/AS160 signaling without effects on body weight in *Lepr*$^{db/db}$ mice following intraperitoneal (i.p.) NPC43 treatment. Male *Lepr*$^{db/db}$ mice at 35 days of age were i.p. injected with 0.2% (v/v) dimethyl sulfoxide (DMSO)/saline (referred to Saline) or NPC43 (0.136 milligram per kilogram body weight) for 30, 58 and 86 days. **(a)** Decreased blood glucose levels in NPC43-treated *Lepr*$^{db/db}$ mice. Blood glucose levels in 4-hour fasted *Lepr*$^{db/db}$ mice (after i.p. treatment for 30 days) and overnight-fasted animals (after i.p. treatment for 58 and 86 days) were measured using a glucometer. **(b)** Body weight of *Lepr*$^{db/db}$ mice before (Day 0) and after i.p. saline or NPC43 treatment for 86 days. **(c)** Representative Western blots of epididymal white adipose tissue (WAT) proteins isolated from *Lepr*$^{db/db}$ mice after daily i.p. saline or NPC43 treatment for 86 days. WAT proteins (8 μg protein/mouse) from five saline- or NPC43-treated *Lepr*$^{db/db}$ mice were subjected to Western blot analysis using indicated primary antibodies. **(d)** Quantitative changes in protein expression of pInsrβ/Insrβ and pAS160/AS160 in epididymal WAT of *Lepr*$^{db/db}$ mice after i.p. NPC43 treatment for 86 days, as determined by the Western blot analysis shown in panel (c). Quantitative data are presented as

mean ± SEM of indicated number of mice per group. *P* value (the NPC43-treated group vs. the saline-treated group) was determined by performing *Student's t-test*. #*P*=0.059, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, vs. the saline-treated group. In panel (b), *P* values between Saline- and NPC43-treated groups at i.p. day 0 and 86 were 0.35 and 0.33, respectively. In panel (d), *P* values of Insrβ/Gapdh and AS160/Gapdh between Saline- and NPC43-treated groups were 0.41 and 0.31, respectively.

Activated Insr/AS160 signaling in WAT of *Lepr*$^{db/db}$ mice after oral NPC43 treatment

Western blot analysis showed that protein expression of pInsrβ at Y1146 (another marker of activated Insr [6]) and pAS160 at T642, but not total Insrβ or AS160 protein, was visibly increased in epididymal WAT of *Lepr*$^{db/db}$ mice after oral NPC43 treatment (*vs.* the 0.5% CMC group, Figure 2a). Quantitative analysis showed that there was a significant 3.3- and 2.2-fold increase in the ratio of pInsrβ and pAS160 protein levels to total Insrβ and AS160 protein levels, respectively, in the WAT of NPC43-treated *Lepr*$^{db/db}$ mice (*vs.* the 0.5% CMC group, Figure 2b). These results indicated that oral NPC43 treatment was also capable of activating Insr/AS160 signaling in the WAT of *Lepr*$^{db/db}$ mice.

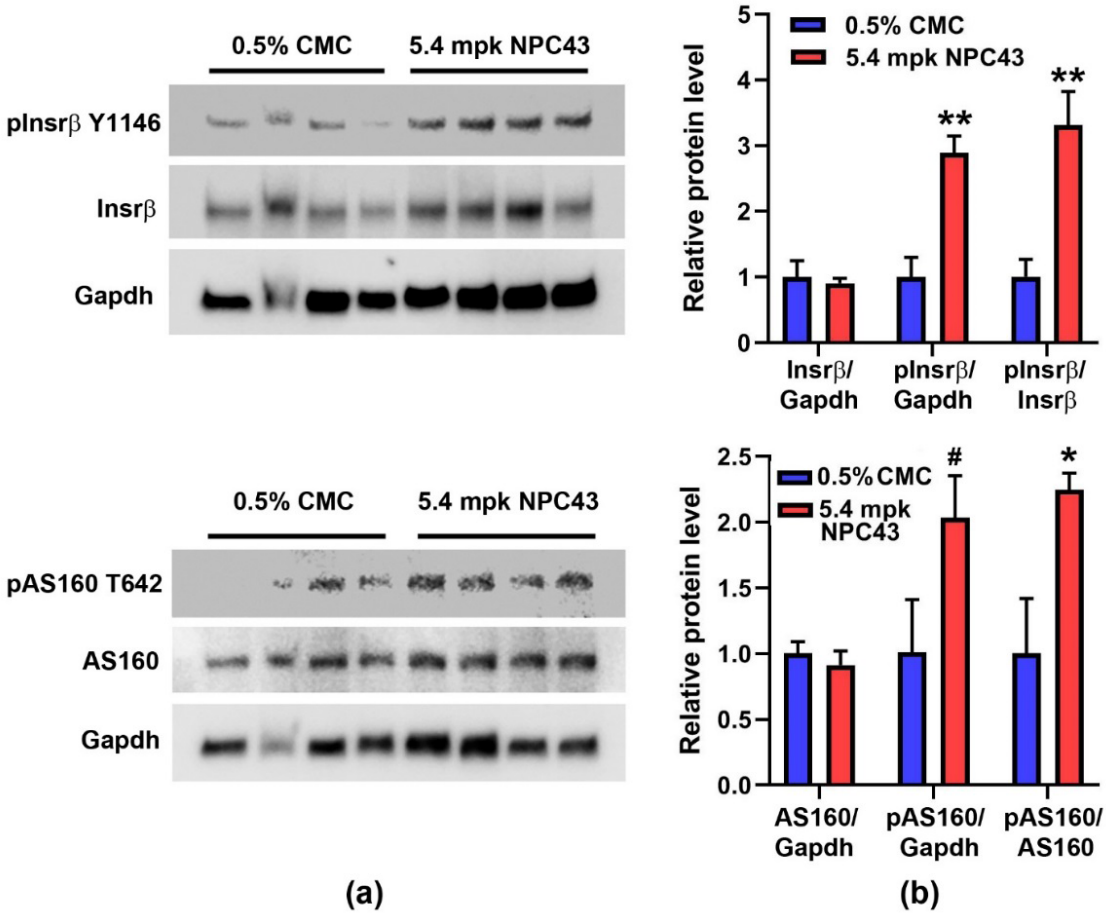

Figure 2. Activation of Insr and stimulation of AS160 phosphorylation in epididymal white adipose tissue (WAT) of *Lepr*$^{db/db}$ mice by NPC43. Male *Lepr*$^{db/db}$ mice at postnatal day 65 were fed daily (by oral gavage) with 0.5% (w/v) carboxymethylcellulose (CMC) or NPC43 [5.4 milligrams per kilogram body weight (mpk)] for 9 days. Epididymal WAT proteins (8 μg protein/mouse) isolated from four CMC- or NPC43-treated *Lepr*$^{db/db}$ mice were subjected to **(a)** Western blot analysis using the indicated antibodies. **(b)** Quantitative changes in protein expression of pInsrβ-Y1146/Insrβ and pAS160-T642/AS160 in epididymal WAT of *Lepr*$^{db/db}$ mice after oral administration of NPC43, as determined by the Western blot analysis shown in panel (a). Data are presented as mean ± SEM of four mice per group. *P* value (the NPC43-treated group *vs.* the control 0.5% CMC group) was determined by performing *Student's t-test.* #*P*=0.09, **P* < 0.05, ***P* < 0.01, *vs.* the 0.5% CMC-treated group. *P* values of Insrβ/Gapdh and AS160/Gapdh between the 0.5% CMC and NPC43-treated groups were 0.73 and 0.54, respectively.

Activated Insr/AS160 signaling in WAT of STZ-induced T1D mice after oral NPC43 treatment

Western blot analysis showed that protein expression of pInsrβ at Y1146 and pAS160 at T642, but not total Insrβ or AS160, were visibly increased in epididymal WAT of STZ-induced T1D mice after oral NPC43 treatment for 5 minutes (Figure 3a). Quantitative analysis showed that there was a significant 4.9- and 2.5-fold increase in the ratio of pInsrβ and pAS160 protein levels to total Insrβ and AS160 protein levels, respectively, in the WAT of NPC43-treated T1D mice (*vs.* the saline-treated mice, Figure 3b). These results demonstrated that NPC43 (per. os.) can replace insulin and activate Insr/AS160 signaling in the WAT of T1D mice.

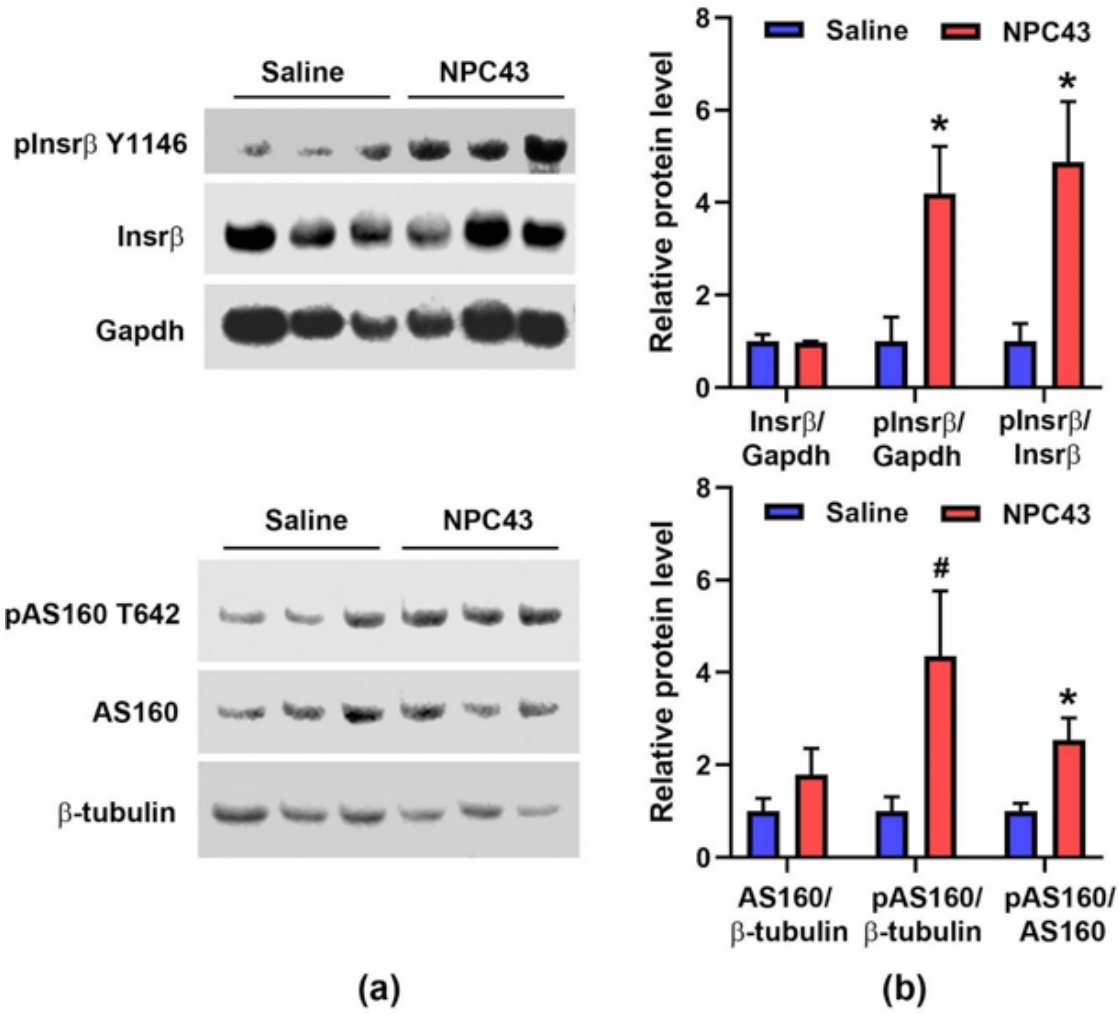

Figure 3. Activation of Insr and stimulation of AS160 phosphorylation in epididymal white adipose tissue (WAT) of streptozotocin (STZ)-induced type 1 diabetic (T1D) mice by NPC43. Four to five-week-old male C57BL6 mice were intraperitoneally injected with STZ [55 milligrams per kilogram body weight (mpk)] daily for 5 days. At 48 days post-STZ treatment, animals were orally administered with 1% (v/v) dimethyl sulfoxide (DMSO)/physiological saline (referred to Saline, n=3) or NPC43 (5.4 mpk, n=3) for 5 minutes. Epididymal WAT proteins (10 μg protein/mouse) isolated from these mice were subjected to **(a)** Western blot analysis using the indicated antibodies. **(b)** Quantitative changes in protein expression of pInsrβ-Y1146/Insrβ and pAS160-T642/AS160 in epididymal WAT of STZ-induced T1D mice after oral administration of NPC43 for 5 minutes, as determined by the Western blot analysis shown in panel (a). Data are presented as mean ± SEM of three mice per group. *P* value (the NPC43-treated group *vs.* the control saline group) was determined by performing *Student's t-test.* $^{\#}P$=0.08, **P* < 0.05, *vs.* the Saline group. *P* values of Insrβ/Gapdh and AS160/β-tubulin between the Saline- and NPC43-treated groups were 0.87 and 0.28, respectively.

Activation of Insr/AS160 signaling by NPC43 in differentiated NIH3T3L1-MBX adipocytes

To investigate whether NPC43 can directly activate adipose Insr/AS160 signaling, NIH3T3L1-MBX fibroblast cells were differentiated into adipocytes (Figure 4). These differentiated adipocytes were incubated with 3.8 μM NPC43 or its solvent (control) for 3 minutes and then subjected to Western blot analysis. As shown in Figure 5a, protein expression of pInsrβ at Y1146 and pAS160 at T642, but not total Insrβ or AS160, were visibly increased in differentiated adipocytes following NPC43 treatment. Quantitative analysis showed that there was a significant 3.8- and 8.5-fold increase in the ratio of pInsrβ and pAS160 protein levels to total Insrβ and AS160 protein levels, respectively, in adipocytes after NPC43 treatment (*vs.* the control group, Figure 5b). These results demonstrated that NPC43 can directly activate Insr/AS160 signaling in adipocytes.

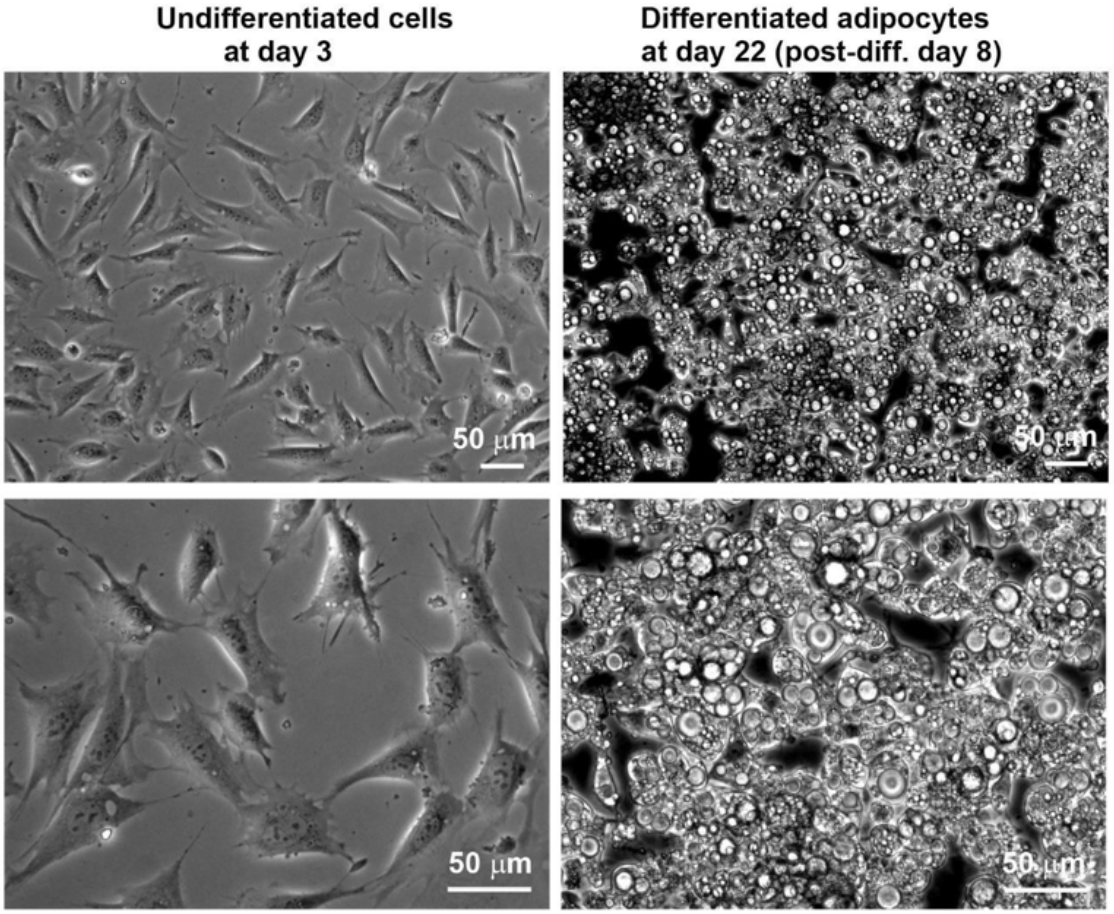

Figure 4. Morphology of NIH3T3L1-MBX fibroblast cells and differentiated mature adipocytes. NIH3T3L1-MBX fibroblast cells, at passage number 3, were seeded on collagen- coated cell culture plates and cultured in Maintenance Medium (97% DMEM, 3% FBS and 1X antibiotic-antimycotic) for 5 days, according to the Promega's Technical Manual of Glucose Uptake-Glo™ assay. These fibroblast cells were then subjected to the differentiation process [via incubation in Differentiation Medium I (DM-I: 97% DMEM, 3% FBS and 1X antibiotic- antimycotic, 1 μg/ml insulin, 0.5 μM isobutylxanthine, 1 μM dexamethasone and 2 μM rosiglitazone) for 7 days followed by Differentiation Medium II (DM-II: 97% DMEM, 3% FBS and 1X antibiotic-antimycotic and 1 μg/ml insulin) for 2 days] and a maturation process (via incubation in Maintenance Medium for 8 days). Images of NIH3T3L1-MBX fibroblast cells (at day 3 of culture) and mature adipocytes at day 22 of culture [also referred to post-differentiation (post-diff.) day 8] were captured under a phase-contrast microscope. Images with a higher magnification are shown in the bottom two panels. The presence of numerous lipid droplets (round particles in right panels) at day 22 of culture indicates the maturity of adipocytes.

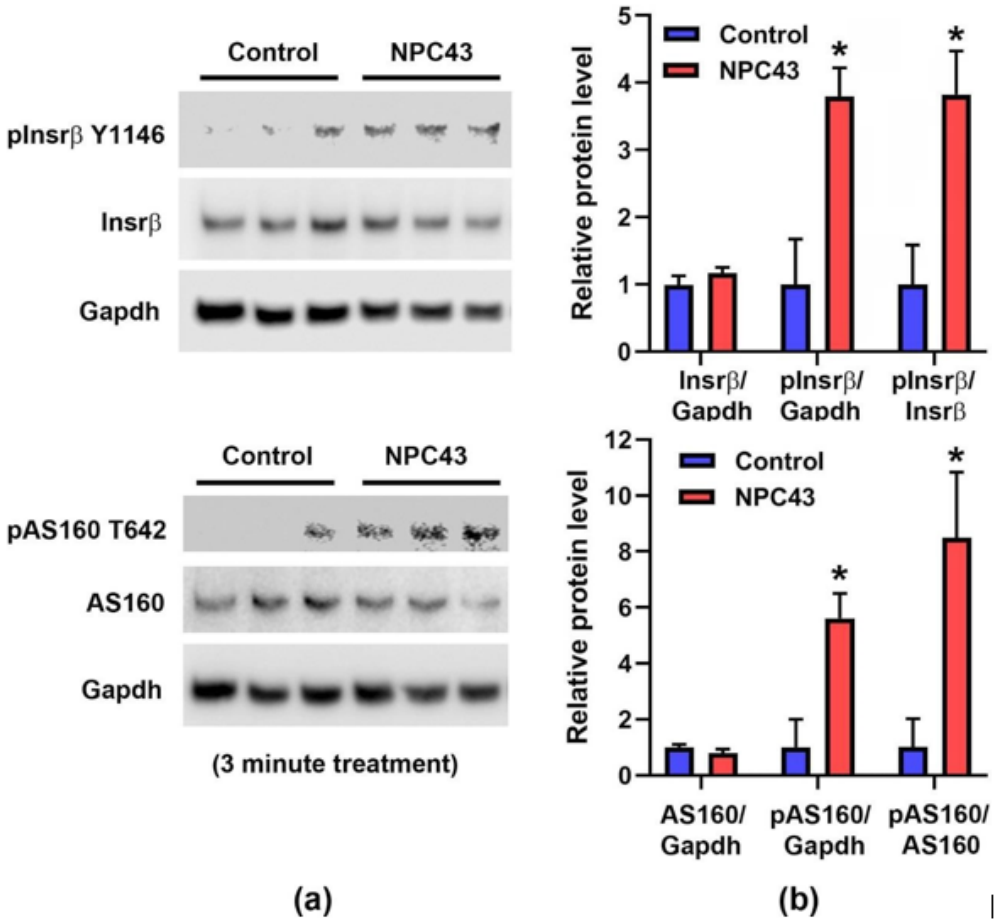

Figure 5. Activation of Insr and stimulation of AS160 phosphorylation in NIH3T3L1-MBX adipocytes by NPC43. NIH3T3L1-MBX fibroblast cells were differentiated into mature adipocytes according to the Promega's Technical Manual of Glucose Uptake-Glo™ assay. Mature NIH3T3L1-MBX adipocytes at post-differentiation day (PD) 8 (equivalent to day 22 of culture) were serum-starved overnight and then incubated with 0.06% DMSO (control) or 3.8 μM NPC43 for 3 minutes. After the above treatments, adipocyte proteins (using 7.5 μg protein/sample) were subjected to **(a)** Western blot analysis using the indicated antibodies. **(b)** Quantitative changes in protein expression of pInsrβ-Y1146/Insrβ and pAS160-T642/AS160 in NIH3T3L1-MBX adipocytes after NPC43 treatment, as determined by the Western blot analysis shown in panel (a). Data are presented as mean ± SEM of triplicate per group. *P* value (the NPC43-treated group *vs.* the control group) was determined by performing *Student's t-test.* **P* < 0.05 *vs.* the control group. *P* values of Insrβ/Gapdh and AS160/Gapdh between the control and NPC43-treated groups were 0.34 and 0.31, respectively.

Enhanced glucose uptake in NIH3T3L1-MBX adipocytes by NPC43 and cooperative action between NPC43 and insulin in adipocyte glucose uptake

The activation, *in vivo* and *in vitro*, of adipose Insr/AS160 signaling by NPC43 as described above, prompted us to investigate the ability of NPC43 in stimulating glucose uptake in NIH3T3L1-MBX adipocytes. As expected, insulin (1 nM) treatment caused a significant increase in glucose uptake (by 182%) in NIH3T3L1-MBX adipocytes [*vs.* the basal control (1st bar in Figure 6)], indicating that the differentiated adipocytes were responsive to insulin. More importantly, a 10.4%, 32.4%, 47% and 66% increase in glucose uptake was observed in adipocytes after treatment with 0.95, 1.9, 3.8 and 7.6 μM NPC43, respectively (*vs.* the control group, Figure 6). Statistical analysis showed that the levels of increased glucose uptake in differentiated adipocytes following 3.8 and 7.6 μM NPC43 treatment were significant when compared to the control group while the extent of increased adipocyte glucose uptake following 1.9 μM NPC43 treatment was close to be statistically significant (*P*=0.07, Figure 6). These results demonstrated that NPC43 can mimic insulin to stimulate adipocyte glucose uptake in a dose-dependent manner.

Cotreatment with 0.1 nM insulin and 0.95 μM NPC43 resulted in a 25% increase, albeit not statistically significant, in adipocyte glucose uptake (*vs.* the control group, P=0.08), which was higher than the 10.4% increase (P=0.29 *vs.* the control group) observed in the 0.95 μM-NPC43 group and the 2% increase (P=0.75 *vs.* the control group) observed in the 0.1 nM-insulin group (Figure 6). A more pronounced and significant increase (81%, *vs.* the control group) in glucose uptake was observed in adipocytes after cotreatment with 1.9 μM NPC43 and 0.1 nM insulin (Figure 6). Similarly, cotreatment with 3.8 μM NPC43 and 0.1 nM insulin resulted in doubling of glucose uptake (101%, Figure 6). These results suggested that NPC43 can act cooperatively with insulin to stimulate glucose uptake in adipocytes.

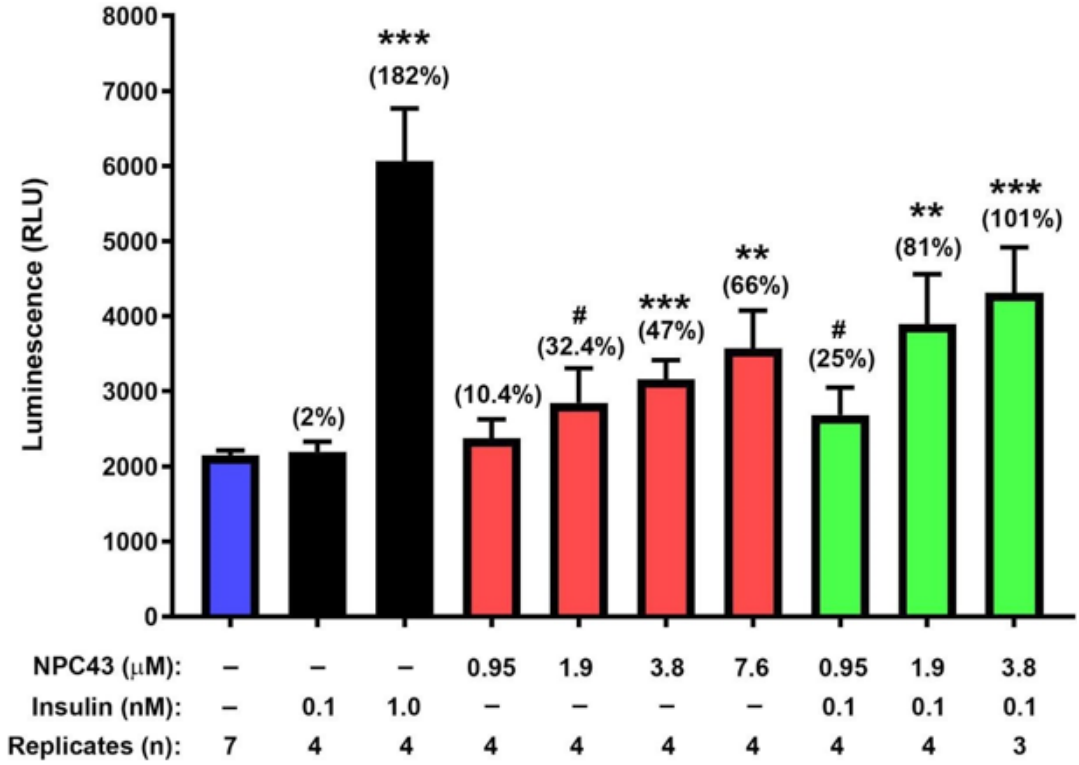

Figure 6. Stimulation of glucose uptake into NIH3T3L1-MBX adipocytes by NPC43 and cooperative action between NPC43 and insulin in adipocyte glucose uptake. Equal number of NIH3T3L1-MBX fibroblast cells (seeded on the collagen-coated 96-well plate) were differentiated to obtain mature adipocytes. Mature NIH3T3L1-MBX adipocytes at post- differentiation day (PD) 7 were pretreated with 0.06% DMSO (NPC43 solvent) or NPC43 (0.95,1.9, 3.8 and 7.6 μM) in serum-free DMEM media overnight. At PD8, those pretreated adipocytes were incubated with 0.06% DMSO (basal control), insulin (0.1 and 1 nM), NPC43 (0.95, 1.9, 3.8 and 7.6 μM), or both insulin (0.1 nM) and NPC43 (0.95, 1.9 and 3.8 μM) in serum- and glucose- free DMEM media at 37°C for 1 hour followed by incubation with 1 μM 2-deoxyglucose (2DG) at room temperature for 10 minutes. Luminescence of up-taken 2DG was determined using a luminometer. Data are presented as mean ± SEM of indicated number of replicates per group. Percentage in parentheses in the bar graph refers to the average percentage of increased glucose uptake in that group when compared to the basal control group. P value [the NPC43- and/or insulin-treated group vs. the basal control group (1st bar)] was determined by performing *Student's t-test*. #$P \leq 0.08$ (but > 0.05), **$P < 0.01$, ***$P < 0.001$, vs. the basal control group.

Discussion

In this report, we demonstrated that i.p. NPC43 treatment can attenuate hyperglycemia in T2D *Lepr*$^{db/db}$ mice (Figure 1a) and both i.p. and oral NPC43 treatment can activate Insr/AS160 signaling in epididymal WAT of *Lepr*$^{db/db}$ mice (Figures 1 and 2). Similarly, activation of Insr/AS160 signaling in epididymal WAT was observed in STZ-induced T1D mice following oral NPC43 treatment (Figure 3). Consistent with these *in vivo* findings, NPC43 was able to directly activate Insr/AS160 signaling in differentiated adipocytes (Figure 5) and stimulate adipocyte glucose uptake in a dose-dependent manner (Figure 6). These findings are consistent with the activation of Insr/AS160 signaling in skeletal muscle and liver tissues previously observed in NPC43-treated T1D and T2D mice [25,26]. Therefore, beside skeletal muscle and liver, WAT is another target tissue of NPC43 in T1D and T2D in which NPC43 can function as an oral or injectable insulin mimetic to activate Insr and its downstream signaling molecule AS160 for glucose uptake.

The decrease in blood glucose levels in *Lepr*$^{db/db}$ mice following i.p. NPC43 treatment (Figure 1a) confirmed the antihyperglycemic activity of NPC43 observed previously [25]. The fasting blood glucose levels (158 μg/dL, Figure 1a) in *Lepr*$^{db/db}$ mice after 86-days of i.p. NPC43 treatment were nearly identical to the blood glucose levels of 135 μg/dL previously found in *Lepr*$^{db/db}$ mice following i.p. NPC43 treatment at the same dose for 90 days [25], indicating that these experiments are very reproducible. It should also be noted that the tested WAT samples in Figure 2 were collected from the same *Lepr*$^{db/db}$ mice receiving oral NPC43 treatment (5.4 mpk daily for 9 days) in which a significant decrease in blood glucose levels was observed [25]. In addition, a significant decrease in blood glucose levels was recorded in STZ-induced T1D mice following oral NPC43 administration [26]. Insr/AS160 signaling is critical for adipose glucose uptake [1,2] and aberrant glucose uptake in adipose tissue is intimately associated with diabetes [4,19]. Thus, the mechanism of NPC43 in lowering blood glucose levels reported here in *Lepr*$^{db/db}$ mice (Figure 1a) and observed previously in T1D and T2D mice [25,26] is due, in part at least, to enhanced blood glucose uptake into WAT as a direct result of the activation of adipose Insr/AS160 signaling by NPC43. Induction of adipose Insr/AS160 signaling by NPC43 in *Lepr*$^{db/db}$ mice (Figures 1 and 2), STZ-induced T1D mice (Figure 3) and differentiated adipocytes (Figure 5), along with enhanced glucose uptake in NPC43-treated adipocytes (Figure 6) support this hypothesis.

It is interesting to note that NPC43 can cooperate with insulin to further enhance glucose uptake in differentiated adipocytes (Figure 6). A similar cooperative action between NPC43 and insulin in stimulating glucose uptake and inhibiting *G6pc* expression (for glucose production) was previously observed in cultured skeletal muscle and liver cells, respectively [25]. It is tempting to infer from these data that NPC43 may also function as an insulin collaborator *in vivo* to restore or potentiate insulin sensitivity by activating Insr to allow glucose uptake in WAT, liver and skeletal muscle, and inhibition of hepatic glucose production in situations of impaired glucose tolerance and the lead up to T2D. In addition, NPC43 likely will be very effective in mitigation of hyperglycemia in diabetic patients with progressive pancreatic β-cells failure.

In STZ-induced T1D mice, protein levels of activated Insr and phosphorylated-AS160 were significantly elevated in epididymal WAT at just 5 minutes after oral NPC43 treatment (Figure 3), which is similar to those reported in skeletal muscle of T1D mice [26]. Consistent with these *in vivo* results, treatment of differentiated adipocytes with NPC43 for just 3 minutes elicited activation of Insr and its downstream signaling molecule AS160 (Figure 5). These results suggest that NPC43 is a fast-acting, oral insulin-mimetic which activates Insr/AS160 signaling in WAT to prevent the onset or progression of diabetes.

A key concern when using insulin mimetics/analogs for the treatment of diabetes is body weight gain [32,33]. However, no significant difference in body weight was observed in NPC43-treated mice relative to saline-treated *Lepr*$^{db/db}$ controls after i.p. treatment for 86 days (Figure 1b). This may represent a further advantage to using NPC43 over insulin or insulin analogs for the treatment of diabetes.

Conclusions

In summary, our studies demonstrated that NPC43 can target WAT to activate Insr/AS160 signaling for glucose uptake and effectively attenuate hyperglycemia in insulin-resistant T2D mice. In addition, NPC43 can mimic insulin to activate Insr/AS160 signaling and stimulate glucose uptake in differentiated NIH3T3L1-MBX adipocytes. Furthermore, the ability of NPC43 in replacing insulin to activate Insr in WAT was observed in the STZ-induced T1D mice. Taken together with our previous studies [25,26], we have a strong body of data to support a role for NPC43 in restoring insulin sensitivity in the most crucial triad of tissues involved in metabolic disorder and the development of T2D. These tissues, skeletal muscle, liver and white adipose are critical for modulating glucose uptake, endogenous glucose production and visceral adiposity, respectively [34]. This, coupled with our findings that NPC43 is effective when administered orally, underscores the potential of this compound as an effective, affordable, non-injectable alternative to insulin. Besides its application in diabetes management, NPC43 may be used for the treatment of a broader range of metabolic disorders/syndromes associated with insulin deficiency and resistance.

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Ethical considerations

Animal studies were pre-approved by Alltech, Inc., and performed according to the U.S. National Institute of Health's Animal Welfare guidelines.

Conflicts of Interest: Zhenmin Lei, Xian Li and Zhiyi Zhao have no conflict of interest. KE and TRY are former Alltech employees while all other authors are current employees of Alltech, Inc. SB is a visiting scholar at Alltech, Inc.

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