A novel SIRT1 activator E6155 improves insulin sensitivity in type 2 diabetic KKA$_y$ mice

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Abstract

Sirtuin 1 (SIRT1) is an NAD$^+$-dependent protein deacetylase that plays a critical role in controlling energy metabolism, stress response and aging. Hence, enhancing SIRT1 activity could be a potential therapeutic strategy to treat metabolic diseases such as diabetes. However, pharmacological activators for SIRT1 are scarce to date. In this study, using the optimized high throughput screening, we identified E6155, a piperazine 1, 4- diamide compound, as a new small molecular activator of SIRT1. We further found that E6155 significantly upregulated glucose uptake in cultured mouse liver cells and skeletal muscle cells through increasing SIRT1 deacetylase activity. In type 2 diabetic KKA$_y$ mice, E6155 treatment markedly decreased the level of fasting glucose. Moreover, E6155 improved oral glucose tolerance and insulin tolerance. Euglycemic clamp and the homeostasis model assessment of insulin resistance index showed that E6155 ameliorated the insulin resistance and increased insulin sensitivity in diabetic mice. Mechanistically, we observed that the antidiabetic effects of E6155 were involved in SIRT1 dependent activation of LKB1/AMPK and IRS1/AKT pathways. In conclusion, our findings identified E6155 as a novel SIRT1 activator and suggested that E6155 could be a promising drug candidate for treating insulin resistance and diabetes.

Keywords

SIRT1; AMPK; AKT; glucose metabolism; insulin sensitivity; diabetes

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Conflict of interests

The authors have declared no conflict of interests.

Introduction

Calorie restriction has pleiotropic effects on cell metabolism and extended lifespan in various species[1]. It has been proposed that the beneficial effects of calorie restriction is partially through silent information regulator 2 (Sir2), a NAD^+-dependent histone deacetylase [2]. Sirtuin 1 (SIRT1) belongs to mammals' sirtuins family, which shares the highest sequence homology with yeast silent information regulator-2 (ySir2), is the most extensively studied member of the class III histone deacetylase [3].

Recently, many studies have demonstrated that SIRT1 activation could improve insulin sensitivity and modulate glucose metabolism [4–6]. SIRT1 has been shown to have benefits in improving insulin resistance and regulating insulin-stimulated glucose uptake by AMP-activated kinase (AMPK)-dependent and insulin receptor substrate 1 (IRS1)/AKT-dependent pathways [7, 8]. Accumulating evidence suggested that AMPK activation could ameliorate insulin resistance and prevent the pathologies of type 2 diabetes [9]. In addition, overexpression of SIRT1 could activate liver kinase B1 (LKB1) and subsequently activate AMPK by decreasing lysine acetylation of LKB1, which caused the translocation of LKB1 from the nucleus to the cytoplasm [10]. The IRS1/AKT pathway is important in mediating Glut4 translocation and glucose transport, however, the signaling is impaired in the conditions of insulin resistance [7, 11]. Also, it was demonstrated that resveratrol, a SIRT1 activator, promotes SIRT1 through LKB1-dependent AMPK activation [12] and IRS1/AKT activation by increasing the phosphorylation of IRS1 and AKT [13]. Therefore, SIRT1 was thought to be a promising therapeutic target for the treatment of metabolic disorders and insulin resistance-related diseases.

In this study, through high throughput screening (HTS), we discovered a novel SIRT1 activator E6155, which is a piperazine 1, 4- diamide compound. We further evaluated the beneficial effects of E6155 on glucose metabolism and explored the possible mechanism *in vitro* and *in vivo*. Our findings demonstrated that E6155 improved insulin sensitivity and prevented insulin resistance in KKA_y mouse model of type 2 diabetes, and thus E6155 might be potentially used in treating diabetes caused by insulin resistance.

Materials and methods

Materials

The small-molecule compound library was maintained by the National Laboratory for Screening New Microbial Drugs, CAMS&PUMC (Beijing, China). E6155 for the animal experiments was ordered from J&K Scientific (Beijing, China). 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) was ordered from Invitrogen (MA, USA). EX527 was purchased from Selleckchem (TX, USA). Insulin, Doxorubicin and Palmitate were obtained from Sigma Aldrich (MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (MA, USA).

Cell culture

HepG2 cells, L02 cells and L6 cells were purchased from National Infrastructure of Cell Line Resource (Beijing, China). HepG2 cells, L02 cells and L6 cells were cultured in

DMEM with 10% FBS (v/v) in 37°C, 5% CO_2 incubator. For L6 myoblast differentiation, after the cells met 80% confluence, the medium was changed to DMEM with 2% FBS for 7 days.

HTS assay for SIRT1 activators

In order to identify SIRT1 activators from the synthetic and natural compound library of our laboratory, we established an HTS assay using the purified recombinant human SIRT1 and HTRF SIRT1 assay purchased from Cisbio bioassays (Parc Marcel Boiteux, France). Reactions were conducted in 384-well white-bottom plates which contains 0.025 U purified SIRT1 enzyme, 1 mM dithiothreitol, 150 μM NAD^+, and 6 nM SIRT1-specific deacetylation substrate-d2, DMSO (final concentration 1%, negative control) or a test compound (final DMSO concentration 1%) each well. Enzyme control wells did not contain SIRT1 enzyme and negative control wells did not contain test compound. After mixing the reaction components, plates were incubated at room temperature for 2 h. Then Anti-acetyl MAb-cryptate was added for additional 5 h. Fluorescence at 620 nm and 665 nm of each well was measured using a fluorometric plate reader (PerkinElmer). Sample activity was defined as the percentage of signal increase relative to the signal window, according to the following formula: ratio = (absorbance at 665 nm/absorbance at 620 nm) $\times 10^4$; deacetylation of substrate (%) = $100-$ ($\text{ratio}_{\text{sample}}$ / $\text{ratio}_{\text{Enzyme control}} \times 100$); upregulating activity regulatory= $\text{ratio}_{\text{sample}}$/$\text{ratio}_{\text{negative control}} \times 100\%$. A value 150% was considered to have a potential activation effect. After validated as potential activators, EC_{50} values were calculated by dose-dependent curve using GraphPad Prism 5 software.

Western blotting

Western blotting was performed as previously described [14]. The primary antibodies include Acetyl-p53 (#2570, Cell Signaling Technology), p53 (#2524, Cell Signaling Technology), Phospho-AMPKα (#2531, Cell Signaling Technology), AMPKα (#5832, Cell Signaling Technology), Phospho-AKT (#9271, Cell Signaling Technology), AKT (#4691, Cell Signaling Technology), and GAPDH (#AB2302, EMD Millipore). All western blot experiments were repeated at least three times and representative images were shown.

Glucose uptake assay

Glucose uptake activity of the cells after the treatment of E6155 with or without EX527 was assayed by 2-NBDG as described previously [15]. L02 or L6 myotubes were incubated with E6155 (0.1 μM) with or without EX527 (10 μM) in 37 °C for 18 h. After that, cells were stimulated with 100 nM insulin for 30 min. Then, insulin was replaced with 50 μM of 2-NBDG. After 1h, the cells were washed three times and lysed in lysis buffer for 10 min. The fluorescence intensity of supernatants which containing equal amount of protein were measured using a fluorometric plate reader (PerkinElmer) at 485 nm excitation and 535 nm emission, respectively. Each glucose uptake assay was performed three times.

Glucose homeostasis analysis in type 2 diabetic KKA$_y$ mice

Female KKA$_y$ mice (16-weeks old, 30–35 g) were purchased from the Institute of Laboratory Animal Sciences, CAMS, PUMC. Animal care and experimental procedures

were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the Institute of Medicinal Biotechnology Institute. In all groups, the mice were fed a high-fat diet to render the mice diabetic (fasting blood glucose>11.1 mmol/L). The model group mice were treated with 0.5% carboxymethylcellulose sodium (CMC-Na). E6155 group were treated with E6155 (10 mg/kg) in CMC-Na by oral gavage once daily for 3 weeks. At day 0, 4, 7, 11, 17 and 20 of the treatment, the mice were fasted for 12 h and the fasting blood glucose was measured using the blood samples from the tail vein. At the end of the experiment, the mice were anesthetized and blood samples were collected from the retro-orbital plexus after fasted for 12 h. Serum insulin, glucose, leptin and adiponectin were measured using commercially available kits (Nanjing SenBeiJia, China) according to manufacturers' protocol.

Oral glucose tolerance test (OGTT)

After 2 weeks of E6155 or vehicle treatment, mice were fasted for 6 h but not limited to the water. 2 g/kg body weight glucose was given by gavage and glucose levels were detected at 0, 30, 60, 90 and 120 min after the glucose treatment [16].

Insulin tolerance test (ITT)

After 2 weeks of E6155 or vehicle treatment, another cohort of mice was fasted for 12 h but have free access to water. Then the mice were subcutaneous injected with insulin solution at 0.75 IU/kg body weight. After 0, 40, 90 and 120 min injection, the blood samples from the tail vein of each mouse were used to measure blood glucose levels [17].

Hyperinsulinemic–euglycemic clamp analysis

After E6155 or vehicle treated for 3 weeks, hyperinsulinemic–euglycemic clamp experiments were performed [18]. Mice were fasted for 12 h and anesthetized before the study. The right jugular vein was catheterized the Y shape pipe for the infusion of glucose and insulin. After the adaptation period, human insulin was infused by syringe infusion pump (KD Scientific, USA) at the rate of 20 mIU/kg/min. When the blood glucose levels decreased to below 6.0 mmol/L, infusion of glucose was started and monitored the blood glucose level every 5 min. The glucose infusion rate (GIR) of five time points after the glucose levels maintaining at 6.0 ± 0.5 mmol/L represents the GIR of the steady state and represents whole-body insulin sensitivity.

Statistical analysis

Data are presented as mean $\pm$ SEM and analyzed with 2-tailed, paired *t*-test or One-way ANOVA with appropriate Bonferroni's multiple comparison test using GraphPad Prim 5 software. $p < 0.05$ was considered as statistically significant.

Results

Identification of E6155 as a SIRT1 activator

To identify potential SIRT1 activators, an HTS assay suitable for SIRT1 activator screening was successfully established. Using the established SIRT1 activator HTS assay, we

identified E6155 (Figure 1A) as a positive hit compound among 12,000 compounds. E6155 named (Z)-1-(4-(3-benzylidene-2,3-dihydro-1H-cyclopenta[b]quinoline-9-carbonyl)piperazin-1-yl)ethan-1-one is a piperazine 1,4-diamide compound that has not previously been reported to have any bioactivity. In our assay, we observed that E6155 activated SIRT1 at a maximum deacetylation of substrate (%) value of 93.69% and with an EC_{50} of 0.88 μM (Figure 1B).

p53 is a well-established deacetylation substrate for SIRT1 [19]. To further validate the activity of E6155 in cultured cells, we analyzed p53 acetylation level in HepG2 cells. Expectedly, E6155 decreased the acetylation level of acetylated p53 significantly (Figure 1C). Moreover, the effect of E6155 on p53 deacetylation was blocked by a SIRT1 specific inhibitor EX527 [20] (Figure 1C). These results demonstrate that E6155 is a *bona fide* SIRT1 activator.

E6155 augmented insulin-induced glucose uptake *in vitro*

SIRT1 has beneficial effects on glucose homeostasis and insulin sensitivity [21]. Since glucose uptake and utilization in liver and skeletal muscle was critical for glucose homeostasis *in vivo*, we evaluated the effect of E6155 on glucose uptake in normal human normal liver L02 cells and L6 myotubes.

The glucose uptake assay showed that E6155 significantly increased the glucose uptake in L02 cells and this effect was reduced when cells were simultaneously treated with SIRT1 specific inhibitor EX527 (Figure 1D). L6 myotubes was formed after 2% FBS stimulated for 7 days. Similarly, the glucose uptake in L6 myotubes was markedly increased after E6155 treatment (Figure 1E). Consistent with the result in L02 cells, this upregulation of glucose uptake by E6155 in L6 myotubes could be blocked by SIRT1 specific inhibitor EX527 (Figure 1E).

E6155 improved whole-body glucose homeostasis in type 2 diabetic KKA$_y$ mice

Since E6155 displays augmented glucose uptake in normal liver and skeletal muscle cells, we further asked whether E6155 has positive effects on diabetes *in vivo*. To this end, 16 week-old female KKA$_y$ mice fed a high-fat diet were used to establish the obesity and hyperglycemia model [22]. The mice were administrated with E6155 (10 mg/kg) or vehicle for 3 weeks. As shown in Figure 2A, compared with vehicle control, the fasting blood glucose levels of the mice in E6155 treated group significantly decreased at day 4, 7, 10, 17 and 20.

To further assess whether E6155 regulate glucose homeostasis through improving glucose tolerance and insulin tolerance in type 2 diabetic KKA$_y$ mice, OGTT and ITT were performed after E6155 treatment for two weeks. OGTT data showed that after the glucose injection, E6155 treatment significantly accelerated the glucose metabolism at four time points examined (Figure 2B). The decreased area under curve (AUC) also showed the improved glucose tolerance and glucose homeostasis after E6155 treatment (Figure 2B). Also, another cohort of mice was treated with E6155 or vehicle for two weeks before performing ITT experiment. As shown in Figure 2C, compared with vehicle control, E6155

significantly lowered blood glucose level and reduced AUC after insulin injection, indicating E6155 improves insulin sensitivity *in vivo*.

In addition, at day 21 (the end of E6155 treatment), four mice were randomly selected to perform the hyperinsulinemic– euglycemic clamp experiments. And the rest mice were anesthetized and blood samples were collected from the retro-orbital plexus. At the end of the clamp, compared with vehicle treated mice, GIRs of the clamp were significantly increased in E6155 treated mice which indicating improved insulin sensitivity after E6155 administration (Figure 2D). These findings demonstrate that E6155 controls the glucose homeostasis by improving glucose tolerance and insulin tolerance in type 2 diabetic KKA$_y$ mice.

Then Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) index was calculated to evaluate the insulin resistance effect of E6155. At day 21, the mice were fasted overnight and serum insulin (Figure 3A) and serum glucose (Figure 3B) levels were determined by commercial kits and HOMA-IR index values were calculated. HOMA-IR index in E6155 treated mice was significantly smaller than that in vehicle treated mice (Figure 3C). These results indicate that E6155 improves insulin resistance in diabetic KKA$_y$ mice.

The leptin level in the serum is closely related to insulin resistance and obesity and reflects the severity of insulin resistance [23]. Adiponectin, released from adipocytes, plays an important role in insulin sensitizing, inflammation and cell apoptotic in multiple cell types. The concentration of adiponectin was dramatically decreased in severe metabolic disorders [24]. The increased ratio of leptin/adiponectin indicates the insulin resistance in obesity [25] and type 2 diabetes mellitus [26, 27]. Our data showed that leptin level was significantly decreased while the adiponectin was upregulated after E6155 treatment (Figure 3D–E) at the end of the experiment. Thus the ratio of leptin/adiponectin markedly decreased by the E6155 treatment (Figure 3F). Collectively, these results demonstrate that E6155 significantly improves whole-body glucose homeostasis by ameliorating insulin resistance in type 2 diabetic KKA$_y$ mice.

E6155 enhanced phosphorylation of AKT and AMPK in type 2 diabetic KKA$_y$ mice and cultured cells

LKB1/AMPK and IRS1/AKT pathways are involved in SIRT1 regulated insulin resistance and insulin-induced glucose uptake [7, 8, 13]. To elucidate the antidiabetic effect of E6155, we first examined the total AMPK and phospho-AMPK protein levels in the liver of diabetic KKA$_y$ mice after E6155 treatment. As shown in Figure 4A, E6155 significantly increased liver AMPK activity by upregulating the phosphorylation of AMPK. *In vitro* studies also showed that E6155 significantly activated AMPK by increasing the phosphorylation of AMPK in HepG2 cells (Figure 4B). Then the IRS1/AKT pathway was examined to test whether E6155 administration could affect it or not. It was found that the phosphorylation level of AKT was increased in the liver of mice treated with E6155 (Figure 4A), indicating that E6155 also enhances the activation of the IRS1/AKT signaling pathway *in vivo*. Similarly, in the case of insulin resistance which induced by Palmitate [28] in HepG2 cells, E6155 increased the level of phosphorylated-AKT significantly (Figure 4C). These results

suggested that the antidiabetic effect of E6155 was at least involved in the activation of AMPK-dependent and IRS1/AKT-dependent pathways.

Discussion

Diabetes is a major cause of mortality and disability worldwide. World Health Organization reported that the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 [29]. An accumulating number of studies have demonstrated that SIRT1 activation may improve insulin sensitivity and modulate glucose metabolism [4–6, 30]. Nonetheless, pharmacological and molecular activators for SIRT1 remain lacking. Resveratrol and SRT1720, which could increase SIRT1 activity, have been reported as a promising therapeutics for type 2 diabetes [21]. Therefore, the efforts for identifying SIRT1 activator have raised a lot of interest these years. In the present study, using HTS assay for SIRT1 activators, we have identified E6155, a piperazine 1, 4- diamide compound, as a novel SIRT1 activator that improve glucose homeostasis.

In the present study, in contemplation of discovering SIRT1 activators, we established an HTS assay for SIRT1 activators using human SIRT1 fragments containing N- and C-terminal catalytic regions (160–665 amino acids). To confirm the upregulation of the bioactive compound, we performed the p53 acetylation assay and predictably found that E6155 increased the effect of SIRT1 deacetylation significantly, which was blocked by SIRT1 specific inhibitor. Previous studies showed that SIRT1 could activate AMPK by activate LKB1 through decreasing lysine acetylation of LKB1 [10]. Also, there are several publications showed that SIRT1 activation could improve insulin resistance and regulating insulin-stimulated glucose uptake through activating AMPK and IRS1/AKT pathway [7–9]. Based on these evidences, we assumed that E6155 exerts the effect of improving insulin sensitivity by SIRT1/LKB1/AMPK and SIRT1/IRS1/AKT pathway *in vivo*. Consistently, the activation of AMPK and AKT by E6155 in cultured cells affirmed our hypothesis. Further studies are needed to clarify the importance of SIRT1/AMPK-dependent pathway and SIRT1/AKT-dependent pathway in mediating E6155 beneficial effect on glucose uptake and insulin sensitivity.

In summary, we have identified a novel SIRT1 activator E6155 regulates glucose metabolism *in vitro* and *in vivo*. Our findings also suggest that SIRT1 could be a novel pharmacological target for modulating insulin sensitivity and glucose metabolism related disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- E6155, a piperazine 1, 4-diamide compound, was first found to be a SIRT1 activator.

- E6155 regulated glucose uptake dependent on SIRT1 in L02 and L6 cells.

- E6155 improved glucose tolerance and insulin sensitivity in type 2 diabetic mice.

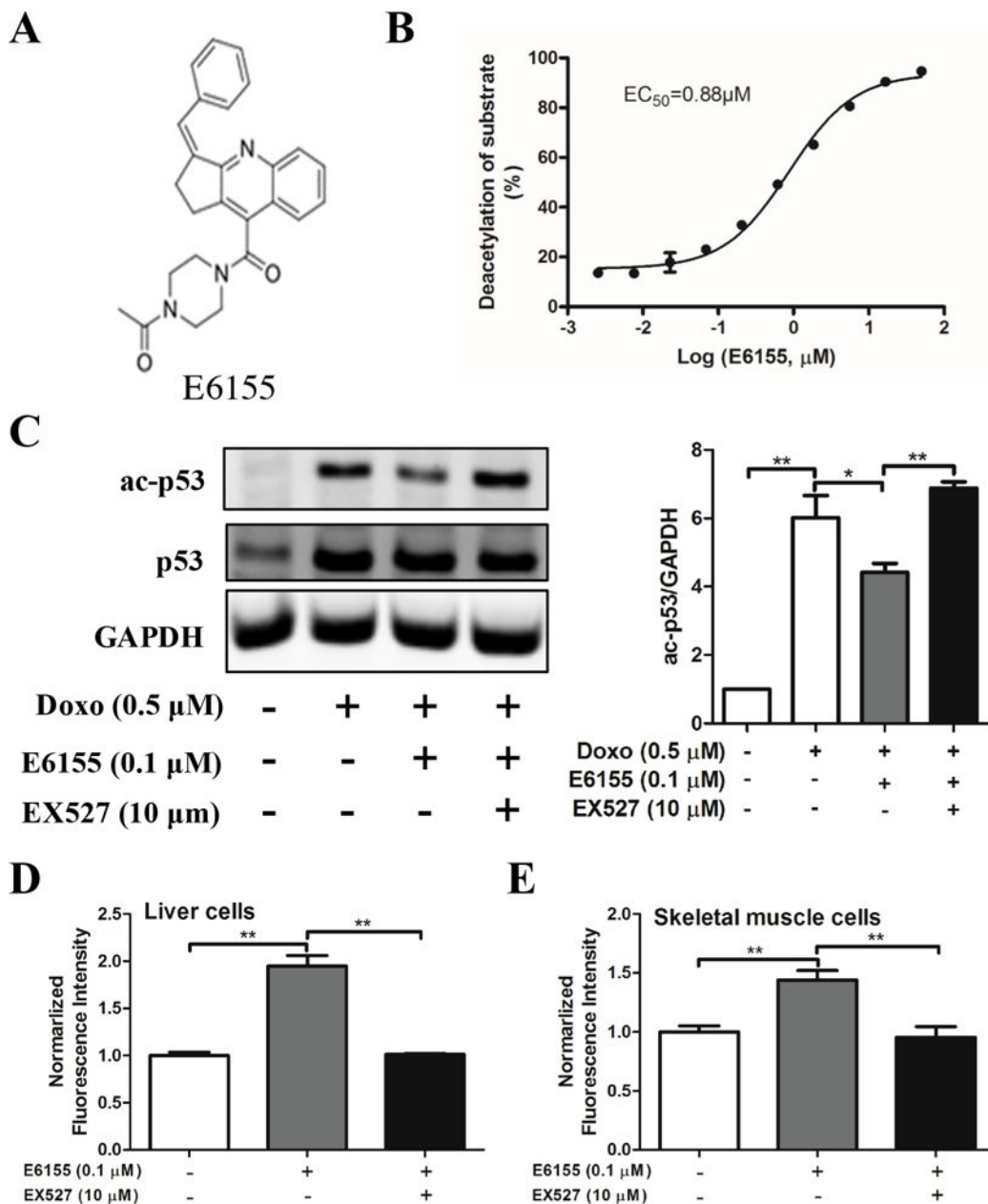- The antidiabetic effect of E6155 was involved in activation of AMPK and AKT pathways.

Figure 1. High throughput screening identified E6155 as a new small molecular activator of SIRT1 and augmented insulin-stimulated glucose uptake

(A) The chemical structure of E6155. (B) Dose-dependent curve of E6155 (in log scale) in SIRT1 activators screening model. (C) Western blotting analysis of ac-p53 and total p53 of whole HepG2 cell lysates. Values are presented as mean±SEM, One-way ANOVA (nonparametric, Bonferroni's multiple comparison test), *p<0.05, **p<0.01, n=4. (D) E6155 improves the glucose uptake in normal liver L02 cells and (E) skeletal muscle L6 cells in a SIRT1 dependent manner. (D–E) Data are expressed as mean ± SEM, 2-tailed, paired *t*-test, *p < 0.05, **p < 0.01, vs Control group, n=3.
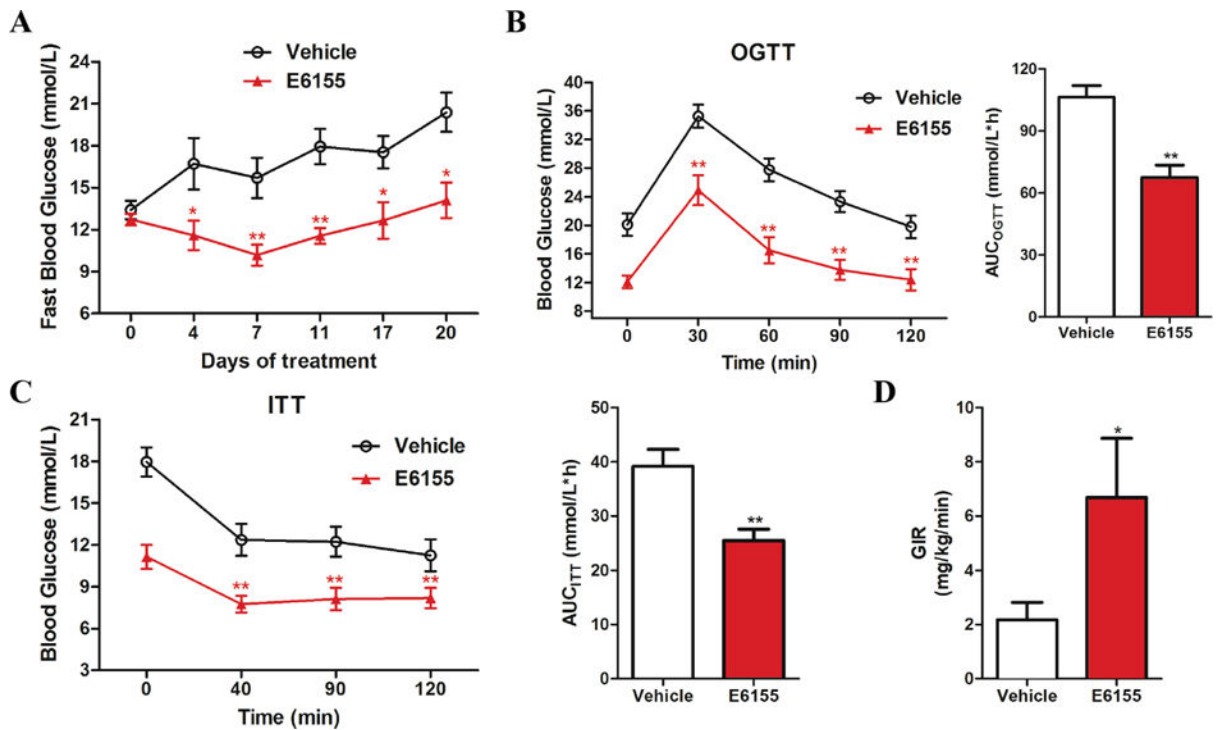
Figure 2. E6155 improved whole-body glucose homeostasis and oral glucose tolerance and insulin tolerance in type 2 diabetic KKA_y mice

(**A**) The fasting blood glucose during the course of treatment with vehicle or E6155. (**B**) Blood glucose levels during the OGTT or (**C**) ITT and the areas under the curve (AUC) of the blood glucose after two weeks of treatment with vehicle or E6155. (**A–C**) Data are expressed as mean ± SEM, 2-tailed, paired t-test, $*p < 0.05$, $**p < 0.01$, vs vehicle group, n=12. (**D**) GIR in Hyperinsulinemic–Euglycemic clamp. Values are expressed as mean ± SEM, 2-tailed, paired t-test, $*p < 0.05$, $**p < 0.01$, vs vehicle group, 4 mice for each group.
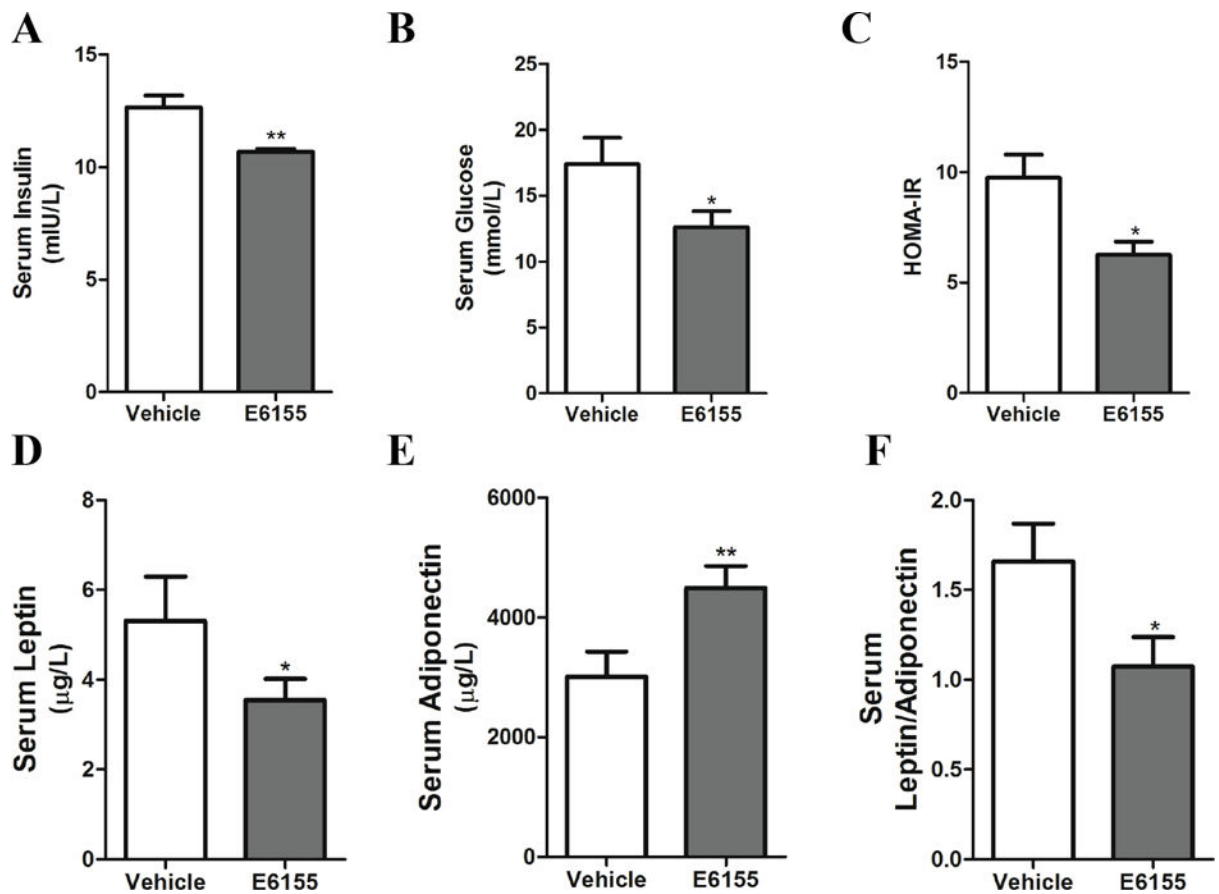
Figure 3. E6155 increased insulin sensitivity in type 2 diabetic KKA$_y$ mice
(**A**) Insulin, (**B**) glucose, (**D**) leptin and (**E**) adiponectin levels in the serum of KKA$_y$ mice administered either vehicle or E6155. (**C**) HOMA-IR index and (**F**) ratio of leptin/adiponectin of vehicle or E6155 group. (**A–F**) Data are expressed as mean ± SEM, 2-tailed, paired *t*-test, *p < 0.05, **p < 0.01, vs vehicle group, n=8.
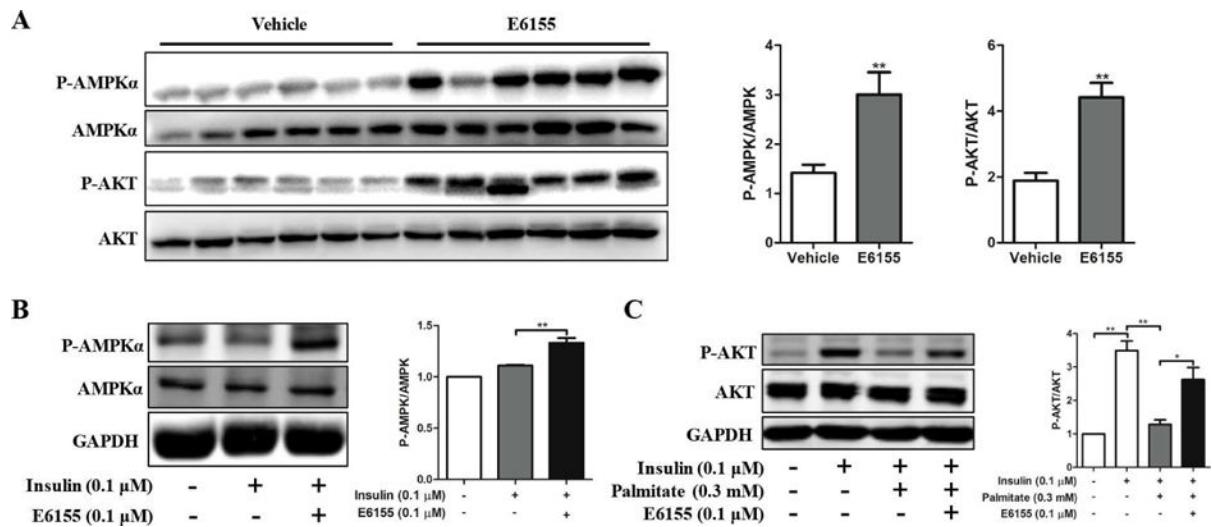
Figure 4. E6155 enhanced phosphorylation of AMPK and AKT in type 2 diabetic KKA$_y$ mice and cultured cells

(**A**) Western Blots analysis of P-AMPKα, AMPKα, P-AKT and AKT in liver from KKA$_y$ mice treated with or without E6155. Representative immunoblots are shown. Values are expressed as means ± SEM, 2-tailed, paired *t*-test, *p < 0.05, **p < 0.01, vs vehicle group, n=12. (**B**) Protein levels of P-AMPKα and AMPKα in HepG2 cells. Cells were pretreated with E6155 (0.1μM) for 6 h, then insulin (0.1μM) was added for 15 min, respectively. (**C**) P-AKT and AKT levels were determined by Western blotting in HepG2 cells. Cells were pretreated with Palmitate (0.3mM) for 18 h, then cells were treated with E6155 (0.1μM) for 6 h, and insulin was added for 15 min finally. (**B–C**) Data are presented as mean±SEM, One-way ANOVA (nonparametric, Bonferroni's multiple comparison test), *p<0.05, **p<0.01, n=3.