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Piceatannol, a resveratrol derivative, promotes glucose uptake through glucose transporter 4 translocation to plasma membrane in L6 myocytes and suppresses blood glucose levels in type 2 diabetic model db/db mice

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ABSTRACT

The skeletal muscle cells are one of the main sites of glucose uptake through glucose transporter 4 (GLUT4) in response to insulin. In muscle cells, 5′ adenosine monophosphate-activated protein kinase (AMPK) is known as another GLUT4 translocation promoter. Natural compounds that activate AMPK have a possibility to overcome insulin resistance in the diabetic state. Piceatannol is a natural analog and a metabolite of resveratrol, a known AMPK activator. In this study, we investigate the *in vitro* effect of piceatannol on glucose uptake, AMPK phosphorylation and GLUT4 translocation to plasma membrane in L6 myocytes, and its *in vivo* effect on blood glucose levels in type 2 diabetic model db/db mice. Piceatannol was found to promote glucose uptake, AMPK phosphorylation and GLUT4 translocation by Western blotting analyses in L6 myotubes under a condition of insulin absence. Promotion by piceatannol of glucose uptake as well as GLUT4 translocation to plasma membrane by immunocytochemistry was also demonstrated in L6 myoblasts transfected with a glut4 cDNA-coding vector. Piceatannol suppressed the rises in blood glucose levels at early stages and improved the impaired glucose tolerance at late stages in db/db mice. These *in vitro* and *in vivo* findings suggest that piceatannol may be preventive and remedial for type 2 diabetes and become an antidiabetic phytochemical.

1. Introduction

The number of diabetic patients is increasing worldwide due to population growth, aging, urbanization, and increasing physical inactivity and prevalence of obesity [1]. To reduce the hyperglycemia, many trials have been conducted before and after glucose absorption from the intestine into bloodstream [2,3]. The skeletal muscles which account for the majority (~80%) of insulin-mediated glucose uptake in the post-prandial state play an important role in maintaining glucose homeostasis [4]. In skeletal muscles, insulin promotes glucose uptake by activation of phosphatidylinositol-3 kinase (PI3K) and Akt, leading to increased translocation of glucose transporter 4 (GLUT4) to the plasma membrane [4]. Another GLUT4 translocation promoter is 5′ adenosine monophosphate-activated protein kinase (AMPK) which is composed of three subunits, α, β and γ [5]. In mammalian cells, AMPK activated by an increase in AMP/ATP ratio acts as an energy sensor [5]. AMPK is activated by exercise/contraction [6] and compounds such as metformin [7], this resulting in stimulation of GLUT4 translocation to plasma membrane and hence glucose uptake in skeletal muscles [8]. Thus, studies on novel compounds that activate AMPK and skeletal muscle glucose uptake would be useful for the development of new treatment of insulin resistance and type 2 diabetes.

Piceatannol (3,5,3′,4′-*trans*-tetrahydroxystilbene), a polyphenol present in grapes and red wine [9], is a natural analog and a metabolite of resveratrol (3,5,4′-*trans*-trihydroxystilbene), and possesses an extra hydroxyl group at the 3′ position of resveratrol. Increasing evidence implies considerable potential of resveratrol to improve health and prevent chronic diseases, but poor bioavailability and rapid metabolism have a possibility to limit the use of resveratrol in dietary intervention for these diseases [10,11]. Resveratrol metabolites such as piceatannol may resolve the problems of poor bioavailability and rapid metabolism and may be an alternative to resveratrol for the health benefit [11]. Our previous findings support this viewpoint; addition of either resveratrol or piceatannol to experimental medium suppressed the proliferation of AH109A hepatoma cells in culture [12,13]. In contrast, addition to experimental medium of serum prepared from rats orally given resveratrol lost the ability to suppress the proliferation of AH109A

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hepatoma cells in culture [12], whereas addition of serum from rats orally given piceatannol maintained the ability to suppress the proliferation [13]. Piceatannol is suggested to have anti-adipogenic and anti-cancer properties [11,13]. However, little is known about the potential effect of piceatannol on AMPK activity and GLUT4 translocation, and hence its effect on the blood glucose level.

In the present study, we have examined whether or not piceatannol induces AMPK activation and GLUT4 translocation *in vitro* employing cultured muscle cells. In addition, we have investigated its effect on hyperglycemia *in vivo* using db/db mice as a model of heavy human type 2 diabetes.

2. Materials and methods

2.1. Materials

A rat skeletal muscle-derived cell line of L6 myoblasts was purchased from American Type Culture Collection (Manassas, VA; ATCC® number: CRL-1458), Dulbecco's modified Eagle medium (DMEM) was from Nissui Pharmaceutical Co. (Tokyo, Japan), fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS), and streptomycin and penicillin G were from Nacalai Tescue, Inc. (Kyoto, Japan). Bovine serum albumin (BSA) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Piceatannol was obtained from Alexis Biochemicals (San Diego, CA). Glucose assay kit (Glucose CII Test Wako) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-phospho-AMPKα (Thr172) and anti-AMPK antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA). Anti-Na^+/K^+-ATPase α-1 antibody was from Milipore (Billerica, MA), anti-GLUT4 antibody was from AbD Serotech (Oxford, UK), horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were from Invitrogen (San Diego, CA). All other chemicals were of the best grade commercially available, unless otherwise noted. Plastic multiwell plates and tubes were obtained from Nunc A/S (Roskilde, Denmark) or Iwaki brand (Asahi Glass Co., Ltd., Tokyo, Japan).

2.2. Determination of glucose uptake by cultured L6 myocytes

Stock cultures of L6 myoblasts were maintained in DMEM supplemented with 10% (v/v) FBS, streptomycin (100 μg/ml), and penicillin G (100 U/ml) (10% FBS/DMEM) as described previously [14]. Effect of piceatannol was examined by the procedure described previously [15] with slight modifications. Briefly, L6 myoblasts (5×10^4 cells/well) were subcultured into Nunc 24-place multiwell plates and grown for 11 days to form myotubes in 0.4 ml of 10% FBS/DMEM. The 11-day-old myotubes were kept for 2 h in Krebs-Henseleit buffer (pH 7.4) containing 0.1% BSA, 10 mM Hepes and 2 mM sodium pyruvate (KHH buffer). The myotubes were thereafter cultured in KHH buffer containing 11 mM glucose without or with piceatannol (0–100 μM) for another 4 h. Piceatannol was dissolved in ethanol and added to KHH buffer at a final ethanol concentration of 0.25%. Glucose concentrations in KHH buffer were determined with a glucose assay kit and a microplate reader (Appliskan, Thermo Fisher Scientific Inc., Waltham, MA) at 508 nm, and the amounts of glucose consumed were calculated from the differences in glucose concentrations between before and after culture. This assay system with L6 myotubes is comparable to that with rat soleus muscles [16] in which L-leucine promoted uptake of [^{14}C]2-deoxyglucose, a GLUT4-binding but non-metabolizable analog of glucose, because L-leucine also stimulated glucose uptake in L6 myotube assay system used in the present study (data not shown).

2.3. Preparation of plasma membrane from L6 myotubes and Western blotting

L6 myoblasts (5×10^5 cells) were subcultured into Nunc 60 mm dishes and grown for 11 days to form myotubes in 3 ml of 10% FBS/DMEM. The 11-day-old myotubes were kept for 2 h in KHH buffer, and then they were cultured in KHH buffer containing 11 mM glucose without or with piceatannol for appropriate time intervals. Plasma membrane fractions were obtained by the methods described by Nishiumi and Ashida [17] with slight modifications as described previously [18]. Cell lysate was prepared from the 11-day-old myotubes, and Western blotting for GLUT4, Na^+/K^+ -ATPase, AMPK, and phospho-AMPK was conducted as described previously [18].

2.4. Construction of HaloTag-glut4 expression vector (pFN21A-rat glut4)

Rat glut4 cDNA is amplified from rat muscle single-strand cDNA (Genostaff Co., Ltd., Tokyo, Japan) using KOD plus DNA Polymerase (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The primer set used for the amplification was the following: 5′-GCGCGATCGCCATGCCGTCGGGTTTCCAG-3′ and 5′-GCGTTTAAACTCAGTCATTCTCATCTGGCCCTAAG-3′. The PCR product was cloned into the *Sgf* I/*Pme* I site of the expression vector pFN21A (HaloTag®7) (Promega KK, Tokyo, Japan). The resulting vector was designated as pFN21A-rat glut4. The construct was verified by DNA sequencing.

2.5. Transfection of the expression vector into L6 myoblasts

L6 myoblasts were used for the transfection and detection of pFN21A-rat GLUT4, because the transfection efficiency into L6 myotube was very low and we could hardly detect the HaloTag® expression (data not shown). To transfect the expression vector and control vector (pFN21A-mock), L6 myoblasts (5×10^4) were cultured in a 24-well culture plate (Nunc) for glucose uptake assay or an 8-well chamber slide (Nunc) for immunocytochemistry. To support cell attachment and growth, an 8-well chamber slide was coated with collagen (Cellmatrix Type I-C, Nitta Gelatin Co., Ltd., Osaka, Japan). After 24 h, at an approximately 60% confluency (visually estimated based on viewing through a microscope), they were transfected with pFN21A-rat glut4 or pFN21A-mock using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. The amounts of transfected DNA were 0.3 μg/well for a 24-well culture plate and 0.2 μg/well for an 8-well chamber slide. Cells were used for the glucose uptake assay at 48 h after transfection and for immunocytochemistry at 36 h after transfection.

2.6. Immunocytochemical staining

At 36 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing twice with PBS containing 0.05% Tween 20 (PBS-T), cells were blocked with 3% non-fat dried skim milk in PBS for 1 h, incubated with anti-HaloTag® rabbit polyclonal antibody (Promega KK, Tokyo, Japan) and anti-caveolin-3 goat polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4 °C. After washing three times with PBS-T, cells were incubated with Alexa Fluor 555-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) and FITC-conjugated anti-goat IgG (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Finally, after washing three times with PBS-T, cells were mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and examined with an Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany).

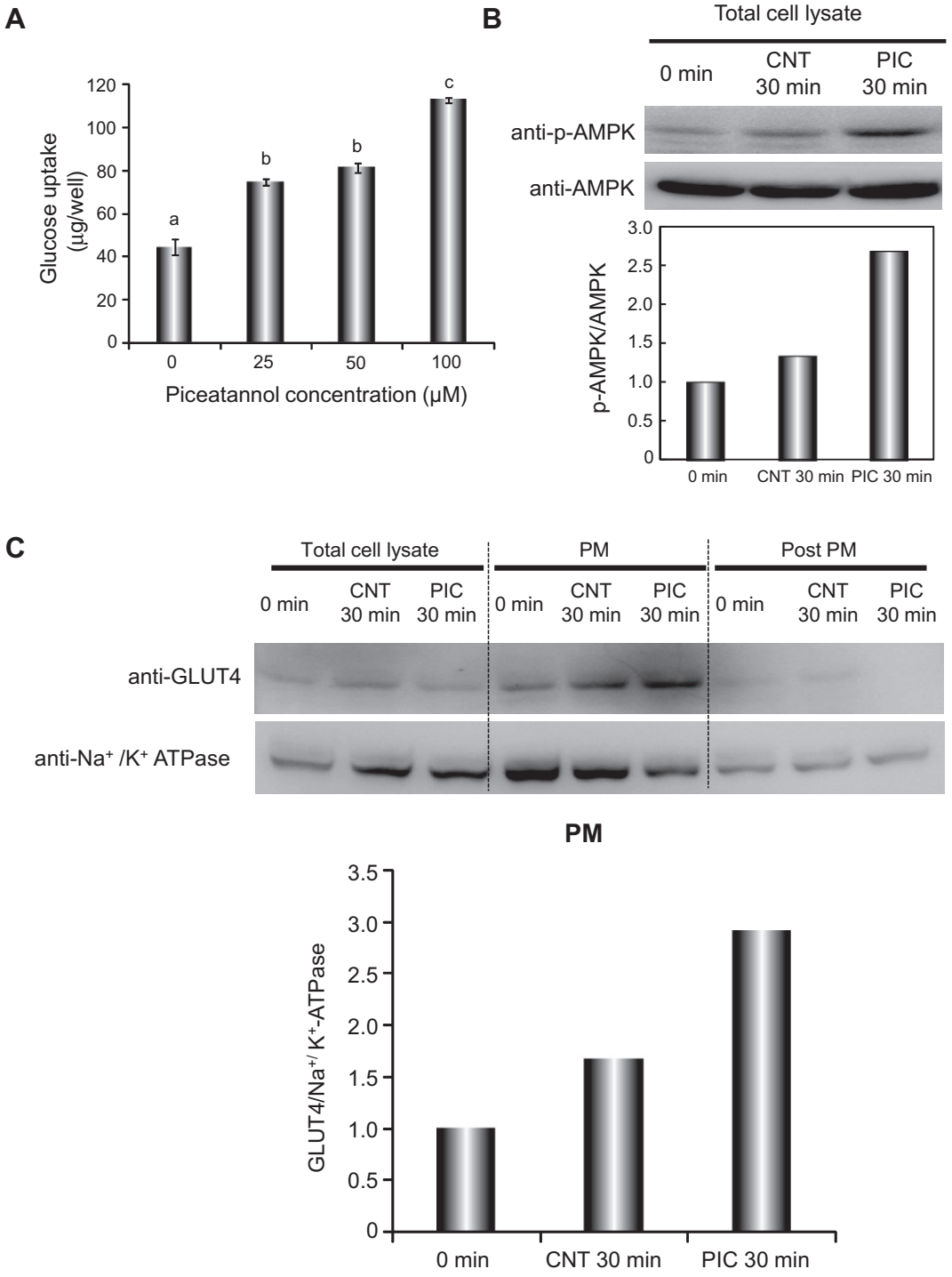

Fig. 1. Effect of piceatannol on glucose uptake (A), phosphorylation of AMPK (B) and translocation of GLUT4 to plasma membrane (C) in cultured L6 myotubes. (A) Glucose uptake for 4 h was measured in 11-day-old L6 myotubes without or with piceatannol (0–100 μM). Each value and bar represents the mean ± SEM of six wells. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test. (B, C) L6 myotubes were treated for 30 min without or with piceatannol (0, 100 μM). Western blotting analyses were conducted with anti-AMPK and anti-phospho-AMPK antibodies (B) and with anti-GLUT4 and anti-Na^+/K^+-ATPase antibodies (C). Protein bands were quantified by ImageJ and ratios of p-AMPK/AMPK (B) and GLUT4/ ATPase in PM fraction (C) are shown. The ratio at 0 min is regarded as 1. Abbreviations: CNT, control; PIC, piceatannol; p-AMPK, phospho-AMPK; PM, plasma membrane fraction; Post PM, post plasma membrane fraction.

2.7. Effect of piceatannol on blood glucose levels in db/db mice

All animal experiments were conducted in accordance with guidelines established by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology and were approved by this committee. Male db/db and its misty (m/m) control (normal) mice (5 weeks of age) were obtained from Charles River Japan (Kanagawa, Japan). Animals were individually housed in stainless-steel cages in an air-conditioned room with an 8:00–20:00 light cycle. All mice were maintained on a stock pellet for

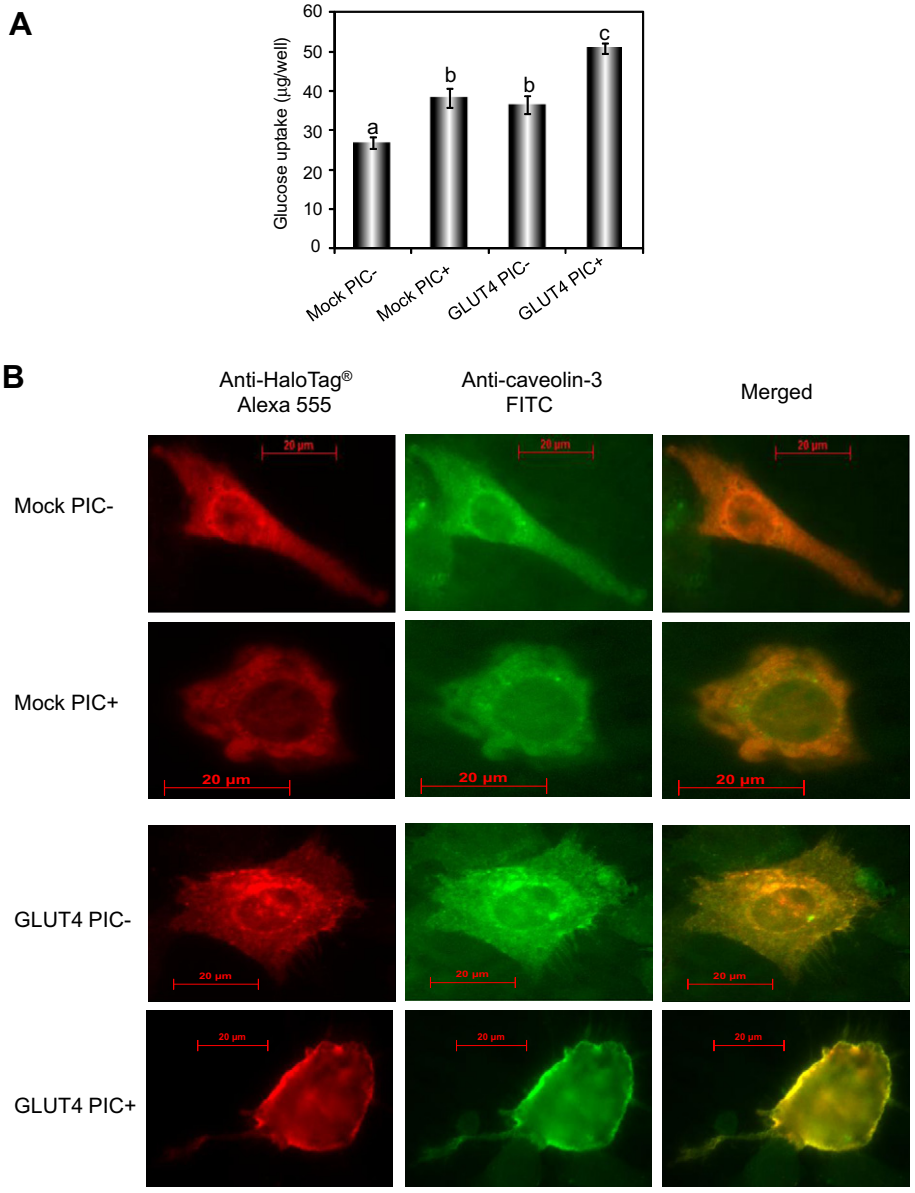

Fig. 2. Effect of piceatannol on glucose uptake (A) and cellular GLUT4 localization by bioimaging (B) in L6 myoblasts transfected with pFN21A-mock vector or pFN21A-glut4 vector. (A) Glucose uptake for 4 h was measured without or with piceatannol (0, 100 μM) in L6 myoblasts 48 h after transfection of pFN21A-mock vector or pFN21A-glut4 vector. Each value and bar represents the mean ± SEM of four wells. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test. (B) L6 myoblasts stably expressing proteins of HaloTag (Mock PIC− and Mock PIC+) or Halo-GLUT4 (GLUT4 PIC− and GLUT4 PIC+) were processed for immunocytochemistry using anit-HaloTag antibody and anti-caveolin-3 antibody. Cellular localization of HaloTag and HaloTag-GLUT4 is shown in red fluorescence (left) and that of caveolin-3 is shown in green fluorescence (center). Merged image is also shown (right). L6 myoblasts (Mock PIC+ and GLUT4 PIC+) were exposed to piceatannol (100 μM) for 30 min.

3 days and thereafter a basal 20% casein diet (20C) as described previously [18]. Piceatannol was suspended in a 0.5% carboxymethyl cellulose sodium salt (CMC, Wako Pure Chemical Industries) aqueous solution at a dose of 5 mg/ml/100 g body weight. Piceatannol suspension or 0.5% CMC alone (vehicle control) (1 ml/100 g body weight) was intubated to mice (6 weeks of age) once a day at 9:00 for 3 weeks, blood was collected 4 h after fasting from tail vein once a week, and glucose concentrations were determined with a kit as described previously [18]. To examine the time-dependent effect of single administration of piceatannol on blood glucose concentrations, two groups of db/db mice (11 weeks of age) that had been maintained on 20C received piceatannol suspension (5 mg/ml/100 g body weight) or 0.5% CMC alone (1 ml/100 g body weight) at 9:00 and they were deprived of their diet but allowed free access to water. Blood was collected 0, 1, 2, 3 and 4 h after oral administrations. Intraperitoneal glucose tolerance test (IPGTT) was also performed using 11-week-old db/db mice, since they showed glucose intolerance [15]. Briefly, two groups of db/db mice (11 weeks of age) were deprived of their diet at 9:00 but allowed free access to water. After fasted for 12 h, piceatannol (5 mg/ml/100 g body weight) and 0.5% CMC alone (1 ml/100 g body weight) were given orally to test and control groups, respectively. Immediately after the oral administrations, blood was collected from the tail vein of mice (0 min). One hour later, diabetic mice received an intraperitoneal injection of glucose (0.2 g/ml/100 g body weight). Blood samples were then successively collected at appropriate time intervals, and blood glucose levels were determined as described above.

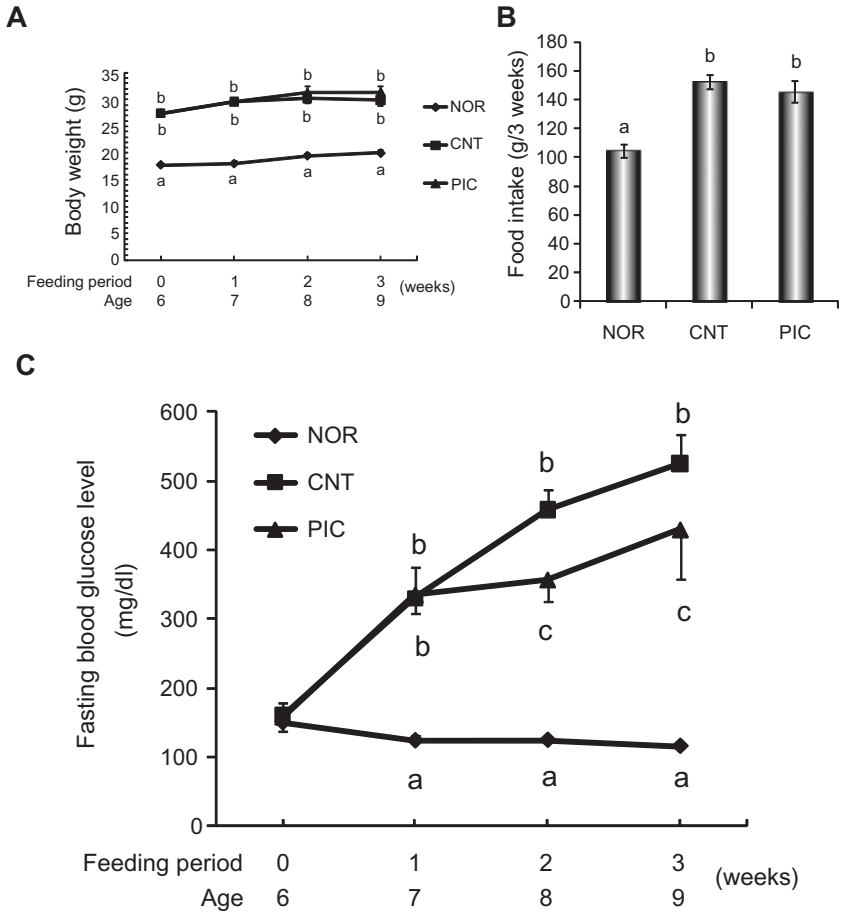

Fig. 3. Effect of piceatannol on rises in fasting blood glucose levels in db/db mice. (A) Changes in body weights. (B) Changes in food intakes. (C) Changes in fasting blood glucose level. Mice were fasted for 4 h before blood collection. Each value and bar represents the mean ± SEM of six (NOR and CNT) or four (PIC) mice. Dose of piceatannol was 5 mg/100 g body weight. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test. Abbreviations: NOR, normal; CNT, control; PIC, piceatannol.

2.8. Statistical analyses

All data are expressed as means ± standard errors of means (SEM). Multigroup comparisons were carried out by one-way analysis of variance followed by Tukey–Kramer multiple comparisons test and differences between two group means were compared by Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Effect of piceatannol on glucose uptake, AMPK phosphorylation and GLUT4 translocation in L6 myotubes

To evaluate the effect of piceatannol on glucose uptake *in vitro*, L6 myotubes were exposed to various concentrations of piceatannol for 4 h, and glucose uptake was calculated by subtracting final glucose concentrations in KHH buffer from initial ones. Piceatannol dose-dependently and significantly promoted glucose uptake in the absence of insulin (Fig. 1A). By exposing L6 myotubes to piceatannol (100 μM) for 30 min, the ratio of phosphorylated AMPK to total AMPK (p-AMPK/AMPK) was demonstrated to increase by Western blotting analysis, indicating piceatannol promoted phosphorylation of AMPK (Fig. 1B). AMPK phosphorylation (=AMPK activation) is known to cause GLUT4 translocation to plasma membrane. Thus, plasma membrane fractions were prepared from L6 myotubes that were exposed to piceatannol (100 μM) for 30 min. Although total amounts of GLUT4 in total cell lysates were similar in control and piceatannol groups, the ratio of GLUT4 to Na^+/K^+-ATPase, a plasma membrane marker enzyme, was found to increase in plasma membrane fractions of piceatannol-treated L6 myotubes. In contrast, GLUT4 was hardly detected in post-plasma membrane fractions, showing the accuracy of fractionation [Fig. 1C]. These findings indicate that piceatannol increases glucose uptake in L6 myotubes through promotion of GLUT4 translocation to plasma membrane *via* AMPK activation.

3.2. Bioimaging of GLU4 translocation in L6 myoblasts transfected with pFN21A-rat glut4 vector

Glucose uptake for 4 h was examined in L6 myoblasts 48 h after transfection of pFN21A-mock vector or pFN21A-glut4 vector (Fig. 2A). Glucose uptake was significantly higher in pFN21A-glut4 vector-transfected cells than in pFN21A-mock vector-transfected ones (Mock PIC− *vs.* GLUT4 PIC−), suggesting that Halo-GLUT4 derived from transfected pFN21A-glut4 could operate in glucose uptake. Piceatannol (100 μM) significantly promoted glucose uptake in L6 myoblasts transfected with both the pFN21A-mock and pFN21A-glut4 vectors (Mock PIC− *vs.* Mock PIC+, GLUT4 PIC− *vs.* GLUT4 PIC+), indicating that piceatannol could promote glucose uptake even under the condition of GLUT4 overexpression. Caveolin-3 is involved in spatial and temporal regulation of GLUT4 translocation to plasma membrane and hence glucose uptake in skeletal muscle cells [19]. Fig. 2B (left) shows cellular localization of HaloT-

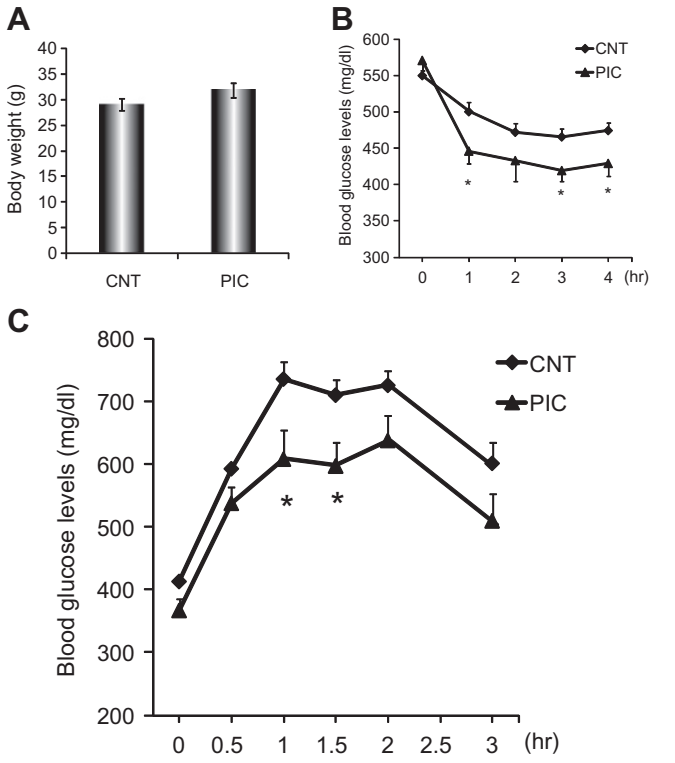

Fig. 4. Effect of piceatannol on impaired glucose tolerance in db/db mice. (A) Body weights of db/db mice (CNT and PIC) at 11 weeks of age. (B) Time-dependent changes in blood glucose levels. (C) Results of IPGTT in db/db mice with glucose intolerance. Dose of piceatannol was 5 mg/100 g body weight (B and C). Each value and bar represents the mean ± SEM of six (CNT) or four (PIC) mice.*$p < 0.05$ *vs.* CNT by Student's *t*-test. Abbreviations: NOR, normal; CNT, control; PIC, piceatannol.

ag protein alone (Mock PIC− and Mock PIC+) and Halo-GLUT4 protein (GLUT4 PIC− and GLUT4 PIC+), Fig. 2B (center) shows cellular localization of caveolin 3, and Fig. 2B (right) shows their merging. In cells transfected with pFN21A-mock vector, HaloTag protein and caveolin-3 expressed in the whole area except for nuclear compartment but did not co-localize (Mock PIC− and Mock PIC+). Likewise, HaloTag protein and caveolin-3 expressed similarly but more strongly expressed in cells transfected with pFN21A-glut4 vector than in cells transfected with pFN21A-mock vector, and co-localization of two proteins was recognized. Piceatannol treatment for 30 min strengthened their co-localization in the plasma membrane compartment as shown by yellowish color (GLUT PIC− *vs.* GLUT PIC+). These findings based on bioimaging method support the promotive effect of piceatannol on GLUT4 translocation to plasma membrane that was demonstrated in biochemical analysis (Fig. 1C).

3.3. Effect of piceatannol on rises in blood glucose levels in db/db mice

Natural compounds that promote glucose uptake in L6 myotubes and activate AMPK have been reported to suppress the rises in blood glucose levels in type 2 diabetic model mice [15,18,20]. The above-mentioned results prompted us to examine *in vivo* effect of piceatannol in younger db/db mice (Fig. 3). Body weights (Fig. 3A), food intakes (Fig. 3B) and fasting blood glucose levels (Fig. 3C) in diabetic control mice were significantly higher than those in normal mice. Piceatannol (5 mg/100 g body weight) suppressed significantly the increases in the fasting blood glucose levels in diabetic db/db mice after treating with piceatannol for 2 and 3 weeks (Fig. 3C) without affecting body weights (Fig. 3A) and food intakes (Fig. 3B). These findings indicate that piceatannol has a potential to prevent the increase in the blood glucose levels at early stages of diabetes in db/db mice. This preventive effect of piceatannol is not due to reduced food intakes, but is thought to be due to its pharmacological action.

3.4. Effect of piceatannol on impaired glucose tolerance in db/db mice

Two groups of db/db mice (11 weeks of age) with the same body weights (Fig. 4A) received a single oral administration of CMC alone (control) and piceatannol (5 mg/100 g body weight), respectively. Piceatannol commenced to significantly reduce the blood glucose level 1 h after administration and the reductive effect was kept up to 4 h later (Fig. 4B). This finding prompted us to conduct IPGTT. As shown in Fig. 4C, piceatannol significantly suppressed the rises in the blood glucose levels 60 and 90 min after its administration. Area under the concentration–time curves (AUC) of blood glucose in the piceatannol group significantly decreased as compared with that in the control group (100 ± 6% for control *vs.* 86 ± 5% for piceatannol, $p < 0.04$). These findings indicate that piceatannol has a remedial effect on impaired glucose tolerance at late stages of diabetes in 11-week-old db/db mice.

In summary, we have found for the first time that piceatannol has potential to prevent and improve type 2 diabetes. One of its modes of action is suggested to be activation of AMPK followed by GLUT4 translocation to plasma membrane of muscle cells that are one of main sites where glucose uptake occurs. Piceatannol could promote AMPK activation, GLUT4 translocation and glucose uptake in cultured muscle cells in the absence of insulin, suggesting this stilbene is capable of overcoming insulin resistance. Although further studies are required to clarify precise mechanisms involved, piceatannol may become a superior candidate phytochemical for prevention and improvement of diabetes.

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