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Mechanisms for antidiabetic effect of gingerol in cultured cells and obese diabetic model mice

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Abstract There have been studies on health beneficial effects of ginger and its components. However, there still remain certain aspects that are not well defined in their anti-hyperglycemic effects. Our aims were to find evidence of possible mechanisms for antidiabetic action of [6]-gingerol, a pungent component of ginger, employing a rat skeletal muscle-derived cell line, a rat-derived pancreatic β-cell line, and type 2 diabetic model animals. The antidiabetic effect of [6]-gingerol was investigated through studies on glucose uptake in L6 myocytes and on pancreatic β-cell protective ability from reactive oxygen species (ROS) in RIN-5F cells. Its in vivo effect was also examined using obese diabetic db/db mice. [6]-Gingerol increased glucose uptake under insulin absent condition and induced 5′ adenosine monophosphate-activated protein kinase phosphorylation in L6 myotubes. Promotion by [6]-gingerol of glucose transporter 4 (GLUT4) translocation to plasma membrane was visually demonstrated by immunocytochemistry in L6 myoblasts transfected with glut4 cDNA-coding vector. [6]-Gingerol suppressed advanced glycation end product-induced rise of ROS levels in RIN-5F pancreatic β-cells. [6]-Gingerol feeding suppressed the increases in fasting blood glucose levels and improved glucose intolerance in db/db mice. [6]-Gingerol regulated hepatic gene expression of enzymes related to glucose metabolism toward decreases in gluconeogenesis and glycogenolysis as well as an increase in glycogenesis, thereby contributing to reductions in hepatic glucose production and hence blood glucose concentrations. These in vitro and in vivo results strongly suggest that [6]-gingerol has antidiabetic potential through multiple mechanisms.

Keywords AMPK · Gingerol · GLUT4 · Immunocytochemistry · L6 myocytes · RIN-5F cells

Abbreviations

AMPK	5′ Adenosine monophosphate-activated protein kinase
GLUT4	Glucose transporter 4
G6Pase	Glucose-6-phosphatase
GS	Glycogen synthase
LGP	Liver glycogen phosphorylase

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PEPCK   Phosphoenolpyruvate carboxykinase
TBARS   Thiobarbituric acid-reactive substances
TC   Total cholesterol
TG   Triglyceride

Introduction

Type 2 diabetes and pre-diabetes are dramatically increasing worldwide. The pathophysiological factors of type 2 diabetes mellitus consist of insulin resistance and pancreatic β-cell failure. Thus, the available anti-hyperglycemic agents related to these causes are necessary to treat and prevent type 2 diabetes (Del Prato and Marchetti 2004). Insulin resistance causes a decrease of glucose uptake and its utilization in insulin sensitive tissues. The majority (∼75 %) of glucose uptake in the post-prandial state is accomplished in the skeletal muscle by the translocation of glucose transporter 4 (GLUT4) toward plasma membrane (Saltiel and Kahn 2001). GLUT4 translocation in muscle cells is known to depend on two divergent signaling pathways. The first pathway involves the binding of insulin to insulin receptor and the subsequent activation of phosphatidylinositol-3 kinase (PI3K) and Akt (Imamura et al. 2003). On the other hand, 5′ adenosine monophosphate-activated protein kinase (AMPK), regarded as cellular energy sensor, has been shown to promote GLUT4 translocation independently of insulin (Huang and Czech 2007).

Hyperglycemia stimulates the endogenous nonenzymatic reaction of glucose with proteins and this reaction results in the generation of advanced glycation end products (AGEs). AGEs interact with certain receptors for them, thus leading to enhanced oxidative stress in various tissues. In particular, an important part of pancreatic β-cell damage related to diabetes is mediated by free radical, suggesting the protection of pancreatic β-cell from reactive oxygen species (ROS) has a crucial role in improving pancreatic β-cell damage (Lin et al. 2012).

Natural plants contain various medicinal compounds for metabolic syndrome including diabetes. Ginger, which is the rhizome of the plant *Zingiber officinale Roscoe*, is widely used in several food products such as soft beverages and also in many types of pharmaceutical formulations (Kubra and Rao 2012). Among the various derivatives of ginger, [6]-gingerol has been identified as, for instance, antioxidant, anti-inflammatory and anti-cancer principle (Dugasani et al. 2010; Yagihashi et al. 2008). Although there have been studies concerned with anti-hyperglycemic and antioxidant effects of ginger and [6]-gingerol (Al-Amin et al. 2006; Chakraborty et al. 2012), there still remain certain aspects that are not well defined in their anti-hyperglycemic effects and mechanisms.

In the present study, we investigated the effect of [6]-gingerol through the studies on molecular mechanisms using cultured rat skeletal muscle cells (L6 myocytes). We also examined whether or not [6]-gingerol would protect pancreatic β-cells from reactive oxygen species (ROS)-induced stress employing cultured pancreatic β-cells (RIN-5F). To confirm anti-hyperglycemic effect of [6]-gingerol in vivo, we also examined its effect on the fasting blood glucose level, glucose intolerance and gene expression of hepatic enzymes related to glucose metabolism in type 2 diabetic model db/db mice.

Materials and methods

Materials

[6]-Gingerol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A rat skeletal muscle-derived cell line of L6 myoblasts and a rat pancreatic β-cell-derived cell line of RIN-5F cells were obtained from American Type Culture Collection (Manassas, VA, USA; ATCC® numbers: CRL-1458 and CRL-2058, respectively). Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS, USA). Streptomycin and penicillin G were from Nacalai Tesque, Inc. (Kyoto, Japan). Bovine serum albumin (BSA, fatty acid free) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glucose, triglyceride (TG) and total cholesterol (TC) assay kits (Glucose CII-Test Wako, Triglyceride E-Test Wako and Cholesterol E-Test Wako, respectively) were from Wako Pure Chemical Industries. Serum lipid peroxide was estimated as thiobarbituric acid-reactive substances (TBARS) with a commercial kit (TBARS Assay Kit), ZeptoMetrix Corporation (Buffalo, NY,

USA). Tumor necrosis factor (TNF)-α assay kit was from Shibayagi Co., Ltd. (Gunma, Japan). Compound C was from Wako Pure Chemical Industries. The anti-phospho-AMPKα (Thr172) and anti-AMPK antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was from Amersham Biosciences (Little Chalfont, UK). Anti-HaloTag® rabbit polyclonal antibody was from Promega KK (Tokyo, Japan). Anti-caveolin-3 goat polyclonal IgG was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Alexa Fluor 555-conjugated anti-rabbit IgG was from Invitrogen (Carlsbad, CA, USA). FITC-conjugated anti-goat IgG was from Santa Cruz Biotechnology Inc. 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).

Determination of glucose uptake by cultured L6 myotubes

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) under an atmosphere of 5 % CO_2/95 % humidified air at 37 °C as described previously (Yagasaki et al. 2003). Effect of [6]-gingerol on glucose uptake was examined by the procedure described previously (Kawano et al. 2009; Ha et al. 2012) with slight modifications. Briefly, L6 myoblasts (5×10^4 cells/well) were cultured in 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 preincubated 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 then incubated in KHH buffer containing 11 mM glucose without or with [6]-gingerol (0–100 µM) for 4 h. [6]-Gingerol was dissolved in dimethyl sulfoxide. Glucose concentrations in KHH buffer were determined with a glucose assay kit, and the amounts of glucose uptake were calculated from the differences in glucose concentrations between before and after incubation with [6]-gingerol.

Western blotting analysis

Western blotting analysis was carried out as described previously (Minakawa et al. 2011). Briefly, L6 myoblasts (5×10^5 cells) were cultured in Nunc 60 mm dishes and grown for 11 days to form myotubes in 3 ml of 10 % FBS/DMEM. The myotubes were incubated for 2 h in KHH buffer, and they were then incubated in KHH buffer containing 11 mM glucose without or with [6]-gingerol for 30, 60, 120 and 240 min. The lysates were subjected to Western blotting analysis for AMPK and phospho-AMPK.

Transfection of pFN21A (HaloTag®7)-glut4 vector into L6 myoblasts and immunocytochemistry

Effect of [6]-gingerol on glucose uptake and GLUT4 translocation was examined by the procedure described previously using HaloTag®-mock vector or HaloTag®-glut4 vector (Minakawa et al. 2012). Briefly, 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. L6 myoblasts were transfected with HaloTag®-mock or HaloTag®-glut4 vector. Cells were used for the glucose uptake assay at 48 h after transfection and for immunocytochemistry at 36 h after transfection. Glucose uptake assay was conducted as mentioned above, and immunocytochemistry was conducted to detect GLUT4 and caveolin-3. Anti-HaloTag rabbit polyclonal antibody and anti-caveolin-3 goat polyclonal antibody were used for the first antibodies. Alexa Fluor 555-conjugated anti-rabbit IgG and FITC-conjugated anti-goat IgG were used for second antibodies.

Synthesis of advanced glycation end product

Artificial advanced glycation end product (AGE) was generated by incubation of BSA with D-glyceraldehyde according to the method of Kume et al. (2005). BSA was incubated at 37 °C for 2 weeks in the presence of D-glyceraldhyde (designated as AGE2). BSA alone was similarly incubated at 37 °C for 2 weeks under conditions without any carbohydrates, and employed as the control for AGE2.

Measurement of intracellular reactive oxygen
species in RIN-5F cells

Intracellular reactive oxygen species (ROS) levels
were measured as described previously (Minakawa
et al. 2011) with slight modifications. Briefly, RIN-5F
cells derived from rat pancreatic β-cells were main-
tained in RPMI 1640 supplemented with 10 % (v/v)
FBS, streptomycin (100 μg/ml), and penicillin G (100
U/ml) (10 % FBS/RPMI 1640) under an atmosphere
of 5 % CO_2/95 % humidified air at 37 °C. The cells
(5×10^5 cells/well) were cultured in Nunc 12-place
multiwell plates. After being cultured for 72 h in 1 ml
of 10 % FBS/RPMI 1640, the medium in each well
was removed. Thereafter, RIN-5F cells received 1 ml
of fresh medium (1 % FBS/RPMI 1640) without or
with [6]-gingerol and AGE2. After the 4 h incubation,
RIN-5F cells were incubated with DCFH-DA in 1 %
FBS/RPMI 1640 at 37 °C for 20 min. At the end of the
incubation, DCFH fluorescence of the cells from each
well was measured at an emission wavelength of
530 nm and an excitation wavelength of 488 nm with
a flow cytometer (EPICS ELITE EPS, Beckman-
Coulter, Hialeah, FL).

Effect of [6]-gingerol 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 old) were obtained from Charles River Japan
(Kanagawa, Japan). Animals were individually
housed in stainless-steel cages with wire bottoms in
an air-conditioned room with a temperature of
22 ± 2 °C, a relative humidity of 60 ± 5 %, and an
8:00–20:00 light cycle. All mice were maintained on a
stock CE-2 pellet diet (CLEA Japan, Tokyo, Japan) for
3 days and thereafter on a basal 20 % casein diet
(20C) for 4 days. The composition of the 20C diet
(AIN-93 formula) was described elsewhere (Minaka-
wa et al. 2011). After preliminary feeding for 1 week,
mice were deprived of their diet at 9:00 but allowed
free access to water until blood collection from tail
vein 3 h later. After determination of blood glucose
concentrations with a commercial kit as described
previously (Minakawa et al. 2011), db/db mice were

divided into two groups of similar fasting blood
glucose levels and body weights (0 week). Diabetic
mice of each of the two groups were given either the
20C as the control (CNT) group or the 20C supple-
mented with 0.05 % [6]-gingerol as the test (GIN)
group for 4 weeks. Likewise, misty mice were given
the 20C as the normal (NOR) group for 4 weeks.
Water and each diet were available at all times except
for the experiments to determine fasting blood glucose
levels, which were carried out every week. After
4 weeks of feeding, whole blood was collected from
all mice by cardiac puncture under somnopentyl
anesthesia. Concentrations of serum lipids, TBARS
and TNF-α were measured with commercial kits.

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was
performed as described previously (Kawano et al.
2009) after the determination of fasting blood glucose
levels at the 3rd week of feeding. Two groups of db/db
mice were deprived of their diet at 20:00 but allowed
free access to water. After fasted for 15 h, [6]-gingerol
(test group) was orally given at a dose of 5 mg/ml of
0.1 % carboxymethyl cellulose sodium salt (CMC)
solution/100 g body weight. Similarly, 0.1 % CMC
solution alone (1 ml/100 g body weight) was orally
given to the control group as a vehicle. Two hours
later, blood was collected from the tail vein of db/db
mice (0 min). Immediately after blood collection,
diabetic mice received an intraperitoneal injection of
glucose (0.2 g/ml/100 g body weight). Blood samples
were successively collected at appropriate time inter-
vals (30, 60, 90 and 120 min), and blood glucose
levels were determined as described above.

Quantitative RT-PCR analyses

Quantitative RT-PCR analyses were conducted as
described previously (Son et al. 2013). Total RNA was
isolated from the liver using modified AGPC method
(Chomczynski and Sacchi 1987). cDNA was synthe-
sized using SuperScript™ III Reverse Transcriptase
kit (Invitrogen, Carlsbad, CA) according to the
manufacturer's instructions. Real-time PCR was per-
formed with FastStart SYBR green Master Mix
(Roche Diagnostics, Mannheim, Germany) in Ther-
mal Cycler Dice® Real Time System II (Takara Bio,
Inc., Tokyo, Japan). The PCR reaction was performed

in duplicate. The relative expression levels of the target genes to the expression level of the endogenous reference gene β-actin were calculated using the delta cycle threshold method. The primer sequences are listed in supplementary Table 1.

Statistical analyses

Data are presented as means ± standard errors of means (SEM). Multigroup comparisons were conducted 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.

Results

Effect of [6]-gingerol on glucose uptake in cultured L6 myotubes

[6]-Gingerol dose dependently and significantly increased glucose uptake in L6 myotubes at concentrations up to 100 μM in the absence of insulin (Fig. 1a). The addition of insulin (100 nM) did not affect glucose uptake in L6 myotubes, indicating that L6 myotubes were insulin-insensitive under our culture conditions. However, the treatment of L6 myotubes with [6]-gingerol alone and combination of insulin and [6]-gingerol significantly increased glucose uptake in the cells (Fig 1b). In many cases, the differentiation to myotubes from myoblasts is performed under a concentration of 2 % FBS like in our previous study (Ha et al. 2012). In the present study, L6 myoblasts were cultured and differentiated to L6 myotubes in the medium containing a higher concentration (10 %) of FBS, because suppliers and hence lots of FBS were different. Previous reports have shown that sustained high glucose and insulin exposure decreases insulin-stimulated GLUT4 translocation in L6 myotubes (Huang et al. 2002). Thus, relatively high concentration of insulin present in FBS might be considered as one of reasons for insulin-insensitivity as seen in Fig. 1b, although precise reasons remain to be elucidated. Compound C, an AMPK inhibitor (Zhou et al. 2001), significantly canceled the [6]-gingerol-induced increase in glucose uptake at a concentration of 10 μM, where compound

C alone exerted no influence on glucose uptake (Fig. 1c). In our preliminary experiment, we have examined the dose-dependent effect of compound C on glucose uptake to find an appropriate concentration where compound C itself exerts no influence, because we considered that the inhibitor alone should not affect glucose uptake in this kind of experiment. As a result, we found that 10 μM of compound C was optimum.

Effect of [6]-gingerol on phosphorylation of AMPK in cultured L6 myotubes

L6 myotubes were treated with 50 μM [6]-gingerol for 30–240 min. [6]-Gingerol stimulated the phosphorylation of AMPK (the ratio of phosphorylated AMPK to total AMPK, p-AMPK/AMPK) from 30 min after treatment, which peaked after 120 min (Fig. 1d).

Effect of [6]-gingerol on glucose uptake in cultured L6 myoblasts transfected with HaloTag®-mock vector or HaloTag®-glut4 vector

Glucose uptake for 4 h was examined in L6 myoblasts 48 h after transfection of HaloTag®-mock vector or HaloTag®-glut4 vector (Fig. 2a). Glucose uptake was significantly higher in HaloTag®-glut4 vector-transfected cells than in HaloTag®-mock vector-transfected cells (mock GIN− vs. glut4 GIN−), suggesting that Halo-GLUT4 protein derived from transfected HaloTag®-glut4 could operate in glucose uptake. [6]-Gingerol (50 μM) significantly promoted glucose uptake in L6 myoblasts transfected with both the HaloTag®-mock and HaloTag®-glut4 vectors (mock GIN− vs. mock GIN+, glut4 GIN− vs. glut4 GIN+), indicating that [6]-gingerol could promote glucose uptake even under the condition of GLUT4 overexpression.

Bioimaging analysis of [6]-gingerol effect on GLUT4 translocation to plasma membrane

To visually observe the translocation of GLUT4 in cells, we employed L6 myoblasts transfected with HaloTag®-glut4 vector, because glucose uptake was higher in L6 myoblasts transfected with HaloTag®-glut4 vector than in those transfected with HaloTag®-mock vector (Fig. 2a). Caveolin-3 is involved in spatial and temporal regulation of GLUT4
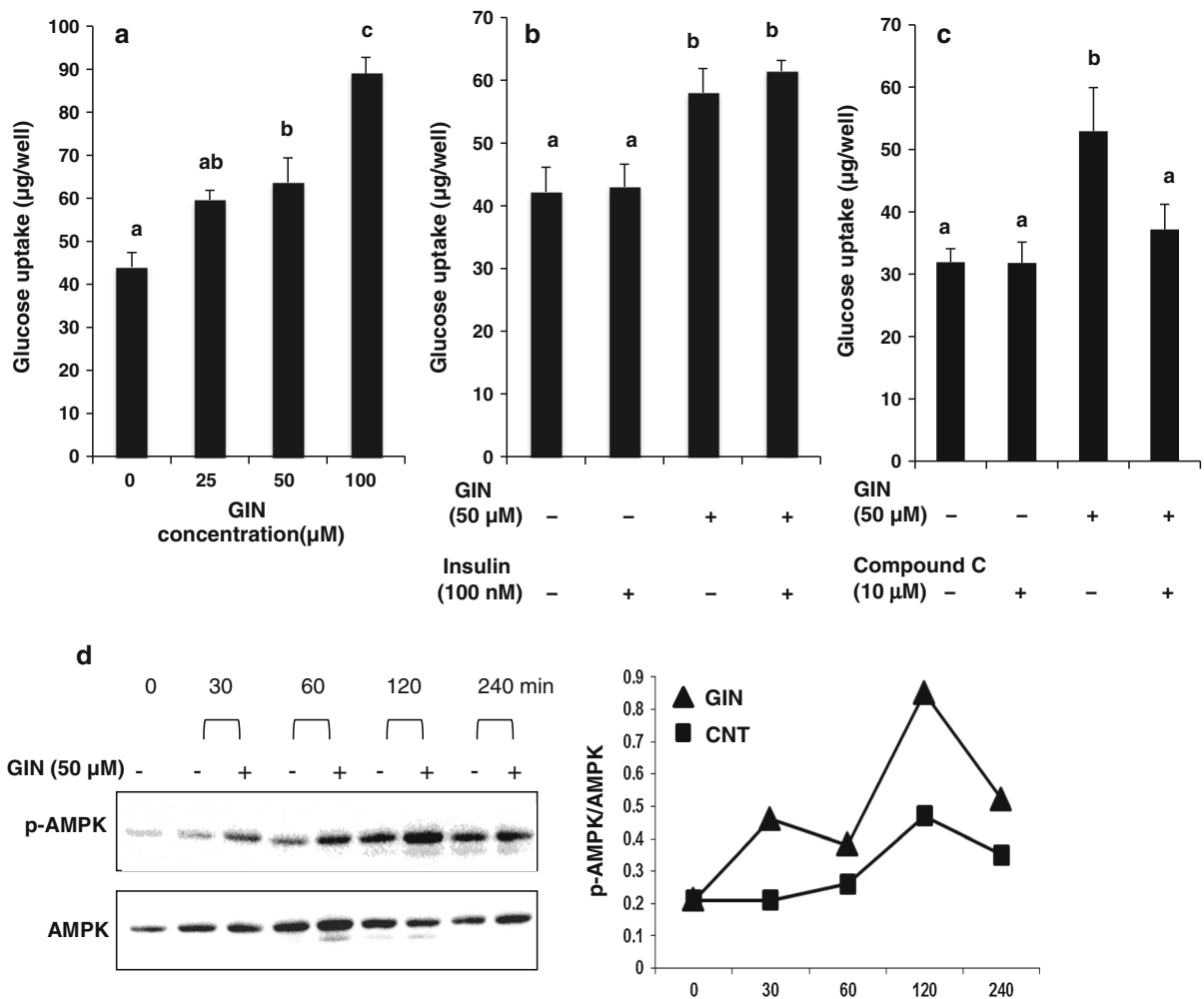
Fig. 1 Effect of [6]-gingerol on glucose uptake and AMPK phosphorylation in L6 myotubes. **a**, **b**, **c** Glucose uptake assay was conducted without or with [6]-gingerol (0, 25, 50 and 100 μM), insulin (100 nM) and compound C (10 μM). Each value represents the mean ± SEM for 6 wells. Values not sharing a *common letter* are significantly different at $P < 0.05$ by Tukey–Kramer multiple comparisons test. **d** Western blotting analysis for AMPK phosphorylation. Western blotting was conducted with anti-phospho-AMPK and anti-AMPK antibodies

translocation to plasma membrane and hence glucose uptake in skeletal muscle cells (Fecchi et al. 2006). Figure 2b (top, red) shows cellular localization of Halo-GLUT4 protein (glut4 GIN− and glut4 GIN+), Fig. 2b (center, green) shows cellular localization of caveolin 3, and Fig. 2b (bottom, yellow) shows their merging. In the absence of [6]-gingerol, Halo-GLUT4 protein and caveolin-3 were expressed in whole area and partially in the cell surface regions in L6 myoblasts transfected with HaloTag®-glut4 vector as shown in Fig. 2b (left, GIN−). [6]-Gingerol treatment at 50 μM for 30 min strengthened co-localization of

GLUT4 protein and caveolin-3 in the plasma membrane compartment as shown by yellowish color in Fig. 2b (right, GIN+).

Changes of intracellular ROS levels in cultured RIN-5F cells by [6]-gingerol treatment

[6]-Gingerol dose-dependently and significantly reduced the basal ROS levels of RIN-5F cells (Fig. 2c) as compared with control cells (0 μM [6]-gingerol). As shown in Fig. 2d, artificial AGE2 was demonstrated to significantly and strikingly enhance the
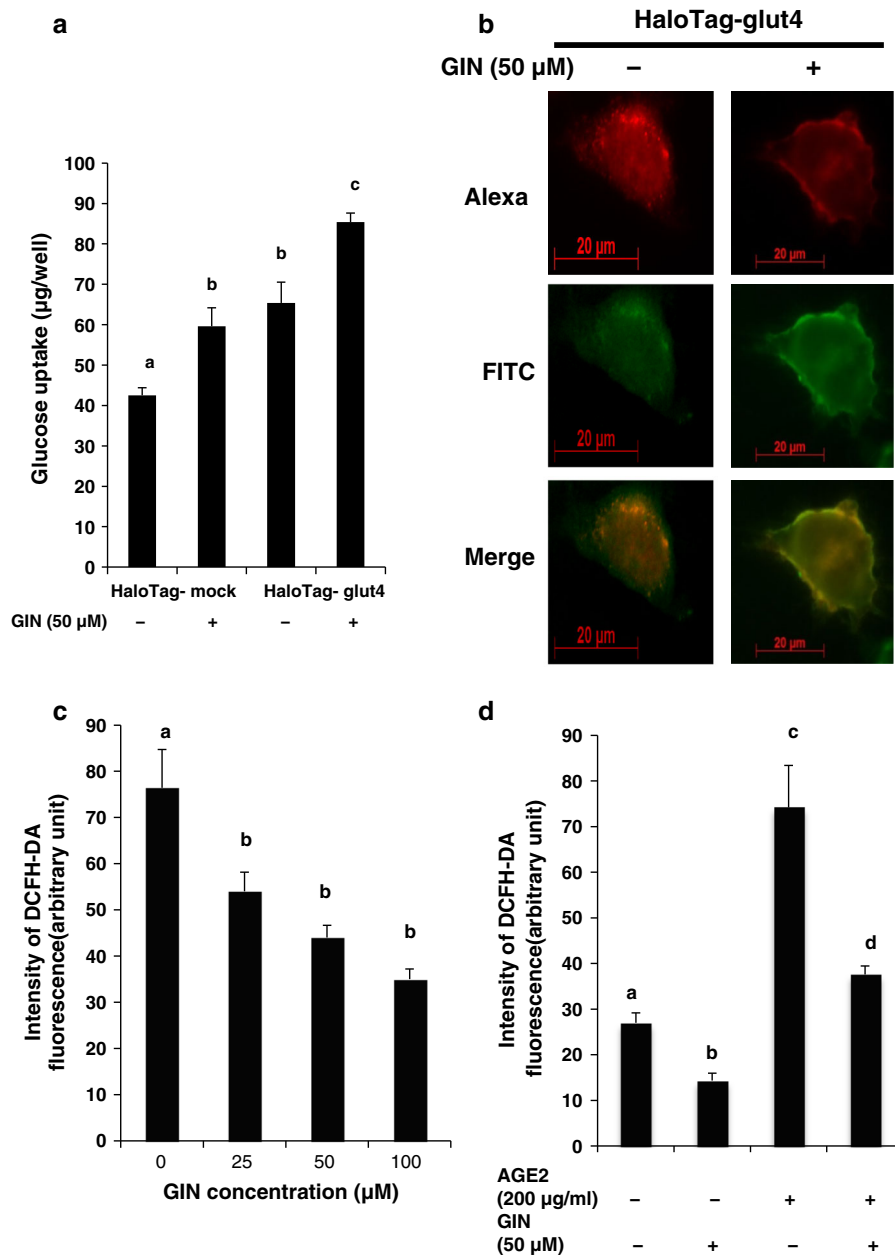
Fig. 2 Effect of [6]-gingerol on glucose uptake and glucose transporter 4 (GLUT4) translocation in cultured L6 myoblasts transfected with HaloTag-mock or HaloTag-glut4 vector and on ROS levels in RIN-5F cells. **a** Glucose uptake in L6 myoblasts transfected with HaloTag-mock or HaloTag-glut4 vector. L6 myoblasts (mock GIN+ and glut4 GIN+) were exposed to [6]-gingerol (50 μM) for 4 h. Each value represents the mean ± SEM for 4 wells. Values not sharing a *common letter* are significantly different at $P < 0.05$ by Tukey–Kramer

multiple comparisons test. **b** GLUT4 translocation in L6 myoblasts transfected with HaloTag-glut4 vector. Immunocytochemistry was processed using anti-HaloTag and anti-caveolin-3 antibodies. **c** Basal ROS levels in RIN-5F cells and **d** AGE2-induced ROS level in RIN-5F cells. Each value represents the mean ± SEM for 3 wells. Values not sharing a *common letter* are significantly different at $P < 0.05$ by Tukey–Kramer multiple comparisons test

production of ROS in RIN-5F cells. However, [6]-gingerol significantly suppressed the AGE2-induced rise in the ROS level.

Effect of [6]-gingerol on fasting blood glucose levels and glucose intolerance in db/db mice

Based on the in vitro results, we examined whether or not [6]-gingerol feeding would improve glucose metabolism in db/db mice. As shown in Fig. 3a, the fasting blood glucose concentrations were significantly higher in control db/db mice (CNT) than in normal mice (NOR). However, [6]-gingerol treatment for 4 weeks significantly suppressed the rise (CNT vs. GIN). In IPGTT, [6]-gingerol significantly suppressed the rise in the blood glucose levels at 60 and 90 min after glucose administration as compared with the CNT group, suggesting that [6]-gingerol could improve glucose intolerance in db/db mice (Fig. 3b). Blood glucose concentrations of diabetic control groups in Fig. 3b (IPGTT) were lower than those in Fig. 3a. This difference might be due to a longer fasting period in Fig. 3b (15 h) than in Fig. 3a (3 h).

Effect of [6]-gingerol on serum levels of lipids and TNF-α in db/db mice

The serum TG, TC, TBARS and TNF-α levels significantly (TC, TBARS and TNF-α) increased or tended (TG) to increase in the CNT group as compared with those in the NOR group, whereas the treatment with [6]-gingerol significantly suppressed these rises (CNT vs. GIN in Fig. 3c–f).

Effect of [6]-gingerol on hepatic gene expression of enzymes related to glucose metabolism

Hepatic gene expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) increased in the CNT group when compared to the NOR group. Treatment of db/db mice with [6]-gingerol significantly suppressed these rises (Fig. 3g, h). Furthermore, the gene expression of glycogen synthase (GS) and liver glycogen phosphorylase (LGP) was significantly lower and higher, respectively, in the CNT group than in the NOR group. In contrast, [6]-gingerol treatment tended to increase the gene expression of GS and significantly suppressed

Fig. 3 Effect of [6]-gingerol on fasting blood glucose levels, glucose intolerance, serum levels of lipids and TNF-α and on hepatic gene expression of enzymes related to glucose metabolism in db/db mice. **a** Fasting blood glucose level. During 4 weeks of [6]-gingerol feeding, fasting blood glucose levels were measured once a week, after mice were fasted for 3 h before blood collection. Each value represents the mean ± SEM for 6 mice. **b** IPGTT test after the determination of fasting blood glucose levels at 3rd week of feeding. After fasted for 15 h, mice of test group (GIN) were given orally [6]-gingerol at a dose of 5 mg/ml CMC/100 g body weight (n = 5), while those of diabetic control group (CNT) were given orally CMC solution alone at a volume of 1 ml/100 g body weight (n = 6). *Asterisk* means statistical significance at $P < 0.05$ by Student's *t* test as compared with CNT group. **c** The levels of serum TG, **d** TC, **e** TBARS, and **f** TNF-α were measured after 4 weeks of [6]-gingerol feeding. The relative mRNA levels of **g** PEPCK, **h** G6Pase, **i** GS and **j** LGP in the liver were measured after 4 weeks of [6]-gingerol feeding. Each value represents the mean ± SEM for 6 mice (**c–j**). Values not sharing a *common letter* are significantly different at $P < 0.05$ by Tukey–Kramer multiple comparisons test. *TG* triglyceride, *TC* total cholesterol, *TBARS* thiobarbituric acid-reactive substances, *TNF-α* tumor necrosis factor-α, *PEPCK* phosphoenolpyruvate carboxykinase, *G6Pase* glucose-6-phosphatase, *GS* glycogen synthase, *LGP* liver glycogen phosphorylase

that of LGP, respectively, as compared with control db/db mice (CNT vs. GIN in Fig. 3i, j).

Discussion

The majority of glucose uptake is accomplished in the skeletal muscle by the translocation of GLUT4 toward plasma membrane (Saltiel and Kahn 2001). In muscle cells, GLUT4 translocation is known to depend on two divergent signaling pathways: insulin signaling pathway and AMPK pathway (Imamura et al. 2003; Huang and Czech 2007). A molecule that promotes glucose uptake in muscle cells through GLUT4 translocation to plasma membrane via AMPK activation is considered to be capable of overcoming insulin resistance (Krook et al. 2004). Thus, we constructed a simple and rapid glucose uptake assay system with L6 myotubes (Kawano et al. 2009) which is comparable to that with soleus muscles of rats and [^{14}C] 2-deoxyglucose (Nishitani et al. 2002). So far, we have found some food components which promote glucose uptake and GLUT4 translocation through AMPK activation in L6 myocytes and possess anti-hyperglycemic effect in type 2 diabetic model mice (Kawano et al. 2009;
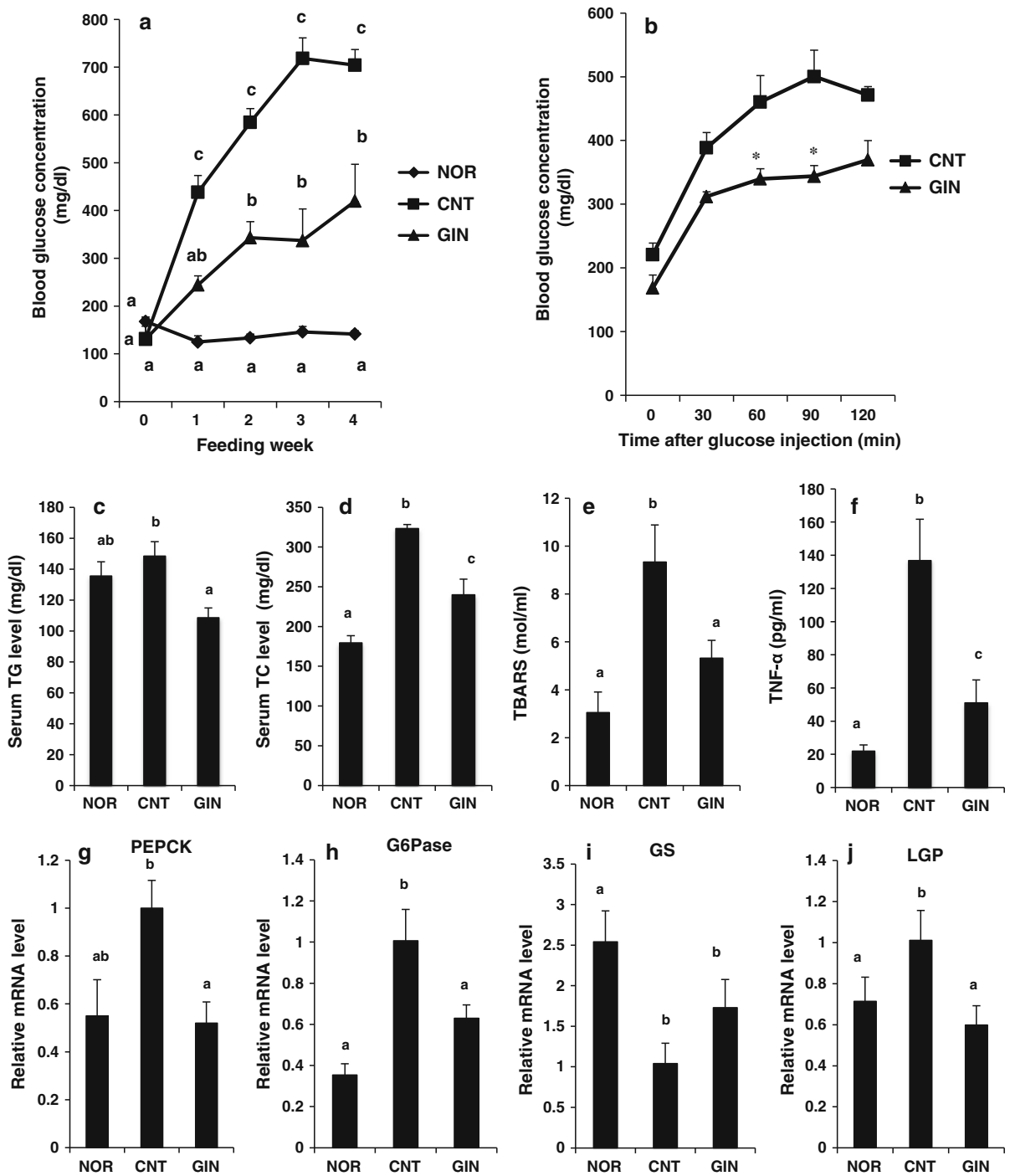
Minakawa et al. 2011, 2012; Ha et al. 2012; Son et al. 2013; Cheong et al. 2014). We have further found [6]-gingerol in the assay system as an antidiabetic candidate molecule in insulin resistant L6 myotubes. Pancreatic β-cells are known to be vulnerable to

oxidative stress (Tiedge et al. 1997), leading to the reduction of insulin secretion. In the present study, we studied modes of stimulatory action of [6]-gingerol on glucose uptake in L6 myocytes and examined whether or not [6]-gingerol, an antioxidant (Dugasani et al.

2010), would protect RIN-5F pancreatic β-cells from oxidative stress. In addition, the effect of [6]-gingerol was studied at the whole body level using type 2 diabetic model db/db mice.

Glucose uptake promoted by [6]-gingerol was entirely eliminated by the addition of compound C. This result suggested that promotion of glucose uptake by [6]-gingerol might depend on the AMPK pathway. Indeed, [6]-gingerol could directly increase AMPK phosphorylation in L6 myotubes. Activation of AMPK has emerged as the critical event that upregulates GLUT4 translocation to the plasma membrane independently of insulin (Krook et al. 2004). Recently, [6]-gingerol has been reported to elevate intracellular Ca^{2+} concentration and subsequently induce an enhancement of phosphorylated AMPKα in L6 myotubes via modulation by Ca^{2+}/calmodulin-dependent protein kinase kinase (CamKK) (Li et al. 2013), this being consistent with the present results. Park et al. (2012) recently show that resveratrol directly inhibits cAMP-dependent phosphodiesterases (PDEs), leading to elevated cAMP levels. The resulting activation of Epac1, a cAMP effector protein, increases intracellular Ca^{2+} levels and activates the CamKK-AMPK pathway via phospholipase C and the ryanodine receptor Ca^{2+}-release channel. As a consequence, resveratrol increases AMPK phosphorylation. Thus, it seems worthy from the aspect of direct target identification to examine whether or not [6]-gingerol could inhibit PDEs.

As was expected from promoted AMPK phosphorylation (= activation) by [6]-gingerol, its treatment strengthened co-localization of GLUT4 protein and caveolin-3 in the plasma membrane compartment of L6 myoblasts transfected with HaloTag®-glut4 vector (Fig. 2b). These results visually verified that [6]-gingerol did promote GLUT4 translocation to plasma membrane in L6 myoblasts, as previously demonstrated for the first time in piceatannol-treated L6 myoblasts as well as L6 myotubes (Minakawa et al. 2012).

Hyperglycemia augments the accumulation of advanced glycation end products (AGEs). It is well recognized that AGEs produce ROS through their receptor and play a significant role in the development of diabetic complications (Wautier and Guillausseau 2001; Fiorentino et al. 2013). The serum level of AGEs is reported to be associated with insulin resistance even in non-obese, non-diabetic subjects

(Tan et al. 2011). Therefore, reducing AGEs-induced ROS as well as the circulating AGEs appears to protect against the development of diabetes and its complications. We examined whether or not [6]-gingerol would protect β-cells from AGEs-induced ROS level using artificial AGE (AGE2). [6]-Gingerol was demonstrated to reduce the artificial AGE2-induced increase in the intracellular ROS level in RIN-5F cells (Fig. 2d). It has been suggested that AGEs can impair insulin sensitivity in skeletal muscle cells and adipocytes (Miele et al. 2003; Unoki et al. 2007). We only have demonstrated that [6]-gingerol has AGE2-induced ROS scavenging activity. Further intensive studies such as measurement of the circulating level of AGEs are required to learn if [6]-gingerol improves diabetic complications caused by AGEs.

[6]-Gingerol feeding to db/db mice was found to suppress the increase in fasting blood glucose concentrations (Fig. 3a). Food intake during the experimental period of 4 weeks was similar between diabetic control and [6]-gingerol-treated db/db mice (data not shown), indicating the suppressive effect of [6]-gingerol on the rise in fasting blood glucose concentrations was not due to reduced food consumption but due to its pharmacological action. [6]-Gingerol administration to db/db mice was also demonstrated to improve glucose intolerance in IPGTT (Fig. 3b). Oral glucose tolerance test (OGTT) has been reported to show similar results in the mice received [6]-gingerol (Singh et al. 2009), suggesting another possibility that [6]-gingerol may act on the intestinal enzyme and/or cells. Further studies are required to clarify this aspect.

Hyperglycemia is particularly related to hepatic glucose synthesis. Excessive hepatic gluconeogenesis is responsible for the high blood glucose level. Thus, suppression of gluconeogenesis in the liver is suggested as an intervention for type 2 diabetes. In the present experiment, [6]-gingerol suppressed the gene expression of hepatic enzymes related to gluconeogenesis (PEPCK, G6Pase) and glycogenolysis (LGP) and tended to enhance the gene expression of hepatic enzyme related to glycogenesis (GS) in db/db mice, like recently demonstrated in ob/ob mice treated with aspalathin (Son et al. 2013) and equol, a metabolite of daidzein (Cheong et al. 2014). Further intensive studies such as measurement of activities of abovementioned hepatic enzymes related to glucose metabolism and identification of direct target of [6]-gingerol

in hepatocytes are required to know precise mechanisms for antidiabetic action of [6]-gingerol.

The serum TG and TC levels increased in diabetic control group as compared with those in normal group. However, [6]-gingerol significantly suppressed these rises, indicating hypolipidemic action of [6]-gingerol, being consistent with other reports in diabetic rats and mice (Al-Amin et al. 2006; Singh et al. 2009). TBARS concentration is widely used as an indicator of lipid peroxidation process and oxidative stress (Bełtowski et al. 2000). [6]-Gingerol also suppressed the rise in the serum TBARS level in db/db mice. In the present study, [6]-gingerol is demonstrated to protect animals from oxidative stress at the whole body level as well as at the cellular level as above-mentioned. From these results, [6]-gingerol is demonstrated to possess its antioxidative function both in vitro and in vivo. Increased level of TNF-α has been reported to be associated with insulin resistance (Winkler et al. 2003). The serum TNF-α level was remarkably higher in diabetic control mice than in normal mice, while [6]-gingerol significantly suppressed this rise. This result suggests a possibility that [6]-gingerol may play a role in reducing insulin resistance in the obese diabetic state.

According to Reagan-Shaw et al. (2008), effective doses in humans are suggested to be one-eighth to one-twelfth of those in mice. Although necessary amounts of [6]-gingerol to apply its hypoglycemic effect in the human body have not been established yet, our research provided the importance and basis of its glucose-lowering effect for further researches in humans as well as animal models.

In summary, [6]-gingerol increased glucose uptake through promotion of GLUT4 translocation via AMPK activation in L6 myocytes. [6]-Gingerol could protect pancreatic β-cells from oxidative stress. The feeding of [6]-gingerol to db/db mice suppressed the increase in the fasting blood glucose level and improved glucose intolerance. [6]-Gingerol was demonstrated for the first time to regulate in vivo hepatic gene expression of enzymes related to glucose metabolism, toward a decrease in glucose production and an increase in glycogen synthesis, these changes contributing to the anti-hyperglycemic effect of [6]-gingerol. Thus, [6]-gingerol may provide therapeutic as well as preventive possibilities for type 2 diabetes.

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