**Effects of Microbial Metabolites of (−)-Epigallocatechin Gallate on Glucose Uptake in L6 Skeletal Muscle Cell and Glucose Tolerance in ICR Mice**

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Glucose uptake ability into L6 skeletal muscle cell was examined with eleven kinds of ring fission metabolites of (−)-epigallocatechin gallate (EGCG) produced by intestinal bacteria. The metabolites 5-(3,5-dihydroxyphenyl)−γ-valerolactone (EGC-M5), 4-hydroxy-5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M6), 5-(3,4,5-trihydroxyphenyl)−γ-valerolactone (EGC-M7) and 5-(3-hydroxyphenyl)valeric acid (EGC-M11) have been found to promote uptake of glucose into L6 myotubes significantly. EGC-M5, which is one of the major ring fission metabolites of EGC-G, was also found to have a promotive effect on glucose transporter 4 (GLUT4) translocation accompanied by phosphorylation of AMP-activated protein kinase (AMPK) signaling pathway in skeletal muscle both in vivo and in vitro. Furthermore, the effect of oral single dosage of EGC-M5 on glucose tolerance test with ICR mice was examined and significant suppression of hyperglycemia was observed. These data suggested that EGC-M5 has an antidiabetic effect in vivo.

**Key words** tea catechin; metabolite;  γ-valerolactone; glucose transporter 4; glucose tolerance

**INTRODUCTION**

Green tea catechins are widely recognized for their various health benefits such as anti-obesity,12 antihypertensive,3,4 prevention of arteriosclerosis,5 lowering of blood cholesterol levels,6 and anti-cancer effects.7–9 On the other hand, intact green tea catechins could not easily be absorbed into the body, especially the most abundant and effective (−)-epigallocatechin 3-O-gallate (EGCG) which is known for its low bioavailability. The absorption rate of EGCG has been reported to be 0.1–1.6% of the oral dose in rats.10 In our previous study, we estimated the bioavailability of intact EGCG, including its conjugates, to be 0.26% after oral administration of [4-3H]EGCG in rats.11 Del Rio et al.12 have reported the bioavailability of tea catechins including EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) would be <4% in humans. Therefore, despite strong activity of EGCG in vitro, the question arises as to whether intact EGCG would show favorable biological activity in the body.

We have investigated EGCG metabolism by rat intestinal microflora and proposed possible metabolic pathways in the intestine.13,14 EGCG was found to be hydrolyzed by intestinal bacteria to yield gallic acid and EGC, subsequently EGC was further degraded to produce some kinds of metabolites in the gut tract. These microbial metabolites were detected in urine after intake of green tea catechin and their bioavailability has been reported to be higher than that of the intact catechins11,12,15 while persisting longer in circulation.11 In our previous report, after oral administration of [4-3H]EGCG to rats, the cumulative amount of microbial metabolites produced in intestine was found to be 26% of the administered dose in rat urine after 24 h.11 Li et al.15 have reported that the human urinary cumulative excretion of microbial metabolites was as high as 8–25 times the levels of EGC and EC. Due to the high bioavailability of ring-fission metabolites in the body, their functions have recently been the focus of intense research as potential contributors to biological activity. However, studies on physiological effects of microbial metabolites are still limited.

Recently, we have reported the isolation and identification of two intestinal bacteria (Adlercreutzia equolifaciens MT4s-5 and Flavonifractor plautii MT42) capable of degrading EGC.14 In the study, we found that A. equolifaciens MT4s-5 catalyzed the conversion of EGC into 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M1) and then F. plautii MT42 converted the propan-2-ol into 5-(3,4,5-trihydroxyphenyl)−γ-valerolactone (EGC-M7) and 4-hydroxy-5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M6) simultaneously. In a similar way, 5-(3,5-dihydroxyphenyl)−γ-valerolactone (EGC-M5) was produced from 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M3), which was formed from EGC by A. equolifaciens MT4s-5 in the presence of hydrogen. These developments made it possible to prepare enough intestinal EGCG or EGC metabolites to be able to examine their physiological activity in vivo.

In many countries hyperglycemia has become a serious health problem of daily life, and with a rapid increase in the number of diabetes mellitus, and type 2 diabetes mellitus patients, many studies have been conducted from the perspectives of prevention and improvement of hyperglycemia using green tea catechin.16–18 Studies conducted in our laboratories have shown that long-term oral dosage of green tea catechins to Goto–Kakizaki (GK) rats with type 2 diabetes, lowered
blood glucose levels as shown by the oral glucose tolerance test.\textsuperscript{19} It has been reported that EGCG improves glucose tolerance level and increases glucose-stimulated insulin secretion after 10 weeks of dietary treatment with AIN-93 diet containing EGCG 1\% (w/w) in genetically diabetic db/db mice.\textsuperscript{20}

As a mechanism for regulating blood glucose concentration, skeletal muscle is a particularly effective target for insulin resistance because skeletal muscle accounts for approximately 80\% of insulin-stimulated glucose uptake in the postprandial state and plays a pivotal role in maintaining glucose homeostasis.\textsuperscript{21} It is reported that insulin-regulated glucose uptake decreases mainly in the skeletal muscle of type 2 diabetes mellitus patients.\textsuperscript{22} Thus, with the goal of improving glucose tolerance and alleviating hyperglycemia, exploration of effective dietary components with the ability to improve glucose uptake into skeletal muscle, has been the focus of this research interest. Glucose transporter 4 (GLUT4) is a major glucose transporter expressing specifically in skeletal and cardiac muscles and adipose tissue, and plays a pivotal role in glucose homeostasis by regulating cellular glucose uptake in these tissues. To induce the uptake of glucose into the muscle cells, insulin stimuli and muscle contraction promote translocation of GLUT4 from intracellular storage vesicles to the plasma membrane. Thus, expression of GLUT4 on the plasma membrane of skeletal muscle is considered to be a target index for improvement of glucose tolerance, as is evaluation of glucose uptake ability.

Concerning EGCG, it has been already reported that EGCG could facilitate the GLUT4 translocation both in skeletal muscle of mice and rats in vitro, and in insulin-resistant L6 myotubes.\textsuperscript{23} Furthermore, EGCG improved glucose uptake by increasing GLUT4 translocation in dexamethasone-induced insulin resistant L6 muscle cells through the activation of both AMP-activated protein kinase (AMPK) and phosphatidylinositol 3 kinase (PI3K)/Akt pathways.\textsuperscript{24} However, we know of no report concerning glucose uptake ability of EGCG microbial metabolites into skeletal muscle and their antidiabetic effect.

In this study, we attempted to evaluate the antidiabetic effect of microbial metabolites produced from EGCG or EGC by intestinal flora. The purpose of this study was to elucidate the potential of microbial metabolites to improve glycemic control in skeletal muscle as the therapeutic target, and to research the possibility that these metabolites could contribute to a hyperglycemic effect in vivo following EGCG consumption. In particular, 5-(3,4,5-trihydroxyphenyl)-γ-valerolactone (EGC-M5), which is the main metabolite produced from EGCG by rat intestinal flora,\textsuperscript{11,13} was investigated mainly for its facilitation of GLUT4 translocation and its underlying molecular mechanism in skeletal muscle cells. Furthermore, anti-hyperglycemic effects of EGC-M5 were confirmed by performing an oral glucose tolerance test in ICR mice.

MATERIALS AND METHODS

Green Tea Catechin and Metabolites (−)-Epigallocatechin gallate (EGCG) was purchased from Sigma-Aldrich Japan Co. LLC. (Tokyo, Japan). The following catechin metabolites were prepared using the methods we reported previously.\textsuperscript{13,14,25} Abbreviation of EGCG metabolites were defined as follows: 1-(3,4,5-trihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (EGC-M1), 4'-dehydroxylated epigallocatechin (EGC-M2), 1-(3,5-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)-propan-2-ol (EGC-M3), 4-hydroxy-5-(3,5-dihydroxyphenyl)-valeric acid (EGC-M4), 5-(3,5-dihydroxyphenyl)-γ-valerolactone (EGC-M5), 4-hydroxy-5-(3,4,5-trihydroxyphenyl) valeric acid (EGC-M6), 5-(3,4,5-trihydroxyphenyl)-γ-valerolactone (EGC-M7), 3-(3,5-dihydroxyphenyl)propionic acid (EGC-M8), 5-(3,5-dihydroxyphenyl)valeric acid (EGC-M9), 5-(3,4,5-trihydroxyphenyl)valeric acid (EGC-M10), and 5-(3-hydroxyphenyl)valeric acid (EGC-M11). Structures of these metabolites and metabolic pathways are illustrated in Fig. 1.

Chemicals and Reagents Glucose-6-phosphate dehydrogenase (G6PDH), 2-deoxyglucose (2-DG) and insulin were purchased from Sigma-Aldrich Japan Co. LLC. Resazurin sodium salt, 5-Amino-4-imidazolecarboxamide-1β-d-ribofuranoside (AICAR), triethanolamine hydrochloride (TEA), and insulin receptor β-subunit (IR) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Diaphorase, ATP and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). For Western blot analysis, anti-IR/β was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Anti-GLUT1, anti-GLUT4, anti-AMPKa, anti-phospho-AMPKa at Thr 172, anti-phospho-P3K at Tyr 458 and Tyr 199, anti-Akt, anti-phospho-Akt at Ser 473 and Thr 308, anti-phospho-PKCζ at Thr 410 and Thr 403, horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) and HRP-conjugated anti-rabbit IgG antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, U.S.A.). Anti-P3K and anti-PKCζ antibodies were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, U.S.A.). All other reagents were used as the highest grade available from commercial sources.

Cell Culture Rat L6 myoblast cells were purchased from Sumitomo Dainippon Pharma Co., Ltd. (Osaka, Japan). L6 myoblast cells were cultured in Eagle’s minimum essential medium (MEM; Nissui Pharma Co., Ltd., Japan) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich Japan Co. LLC.) at 37°C under 5% CO₂ atmosphere. The cells were grown in a 96-well plate for 2-DG uptake assay and 35-mm diameter dish for Western blot analysis. The differentiation of L6 myoblasts to myotubes (4.0×10⁵ cells) was performed according to the previously described method.\textsuperscript{26} The cells were maintained in MEM containing 10% FBS for two days and incubated with MEM containing 2% FBS. Differentiated L6 myotubes were serum starved in MEM containing 0.2% BSA for 18h. Serum-starved L6 myotubes were treated with 0.1, 1, and 3μM EGC-M5 for 15min and subjected to Western blotting.

Glucose Uptake Assay We investigated the insulin-like ability of EGCG metabolites on L6 myotubes. L6 myotubes uptake was determined by enzymatic fluorescence assay for 2-DG uptake in myotubes.\textsuperscript{26} This evaluation was according to the procedure used for other dietary polyphenols.\textsuperscript{27–29} Serum-starved L6 myotubes in a 96-well plate were treated with 11 kinds of metabolites dissolved with dimethyl sulfoxide (DMSO) at a final concentration of 3μM for 4h in 0.2% (w/v) BSA/MEM. Insulin at 100nM was used as a positive control, and DMSO was used as a vehicle control. The treated cells were further incubated with 1mM of 2-DG for 20min in Krebs–Ringer-Phosphate–N-(2-hydroxyethyl)piiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (KRP; 50mM HEPES, 10mM KRP, 8mM NaCl, 0.5mM CaCl₂, 1mM MgCl₂, 1mM Na₂HPO₄, 0.1mM KH₂PO₄, 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin).
pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM CaCl₂, and 1.3 mM MgSO₄) containing 0.1% BSA. Then the cells were washed twice with KRPH buffer containing 0.1% BSA, lysed with 0.1 M NaOH, and dried at 85°C for 50 min. The dried cell lysates were solubilized and neutralized in 0.1 M HCl and 150 mM TEA buffer pH 8.1. The solubilized lysates were incubated with assay cocktail (50 mM TEA, pH 8.1, 50 mM KCl, 0.02% BSA, 0.1 mM NADP, 15 units/mL G6PDH, 0.2 units/mL diaphorase and 2 mM resazurin sodium). The fluorescence of the resorufin produced from resazurin was measured at excitation and emission wavelengths of 530 and 570 nm, respectively, using plate reader Wallac 1420 ARVOsx (Perkin-Elmer, Boston, MA, U.S.A.).

Preparation of Plasma Membrane Fraction Protein in the plasma membrane fraction and whole lysates from cultured cells and muscle tissues were prepared as previously described.²⁶,³⁰) For the plasma membrane fraction, L6 myotubes or muscle tissue were collected with 50 mM Tris buffer, pH 8.0, containing 0.5 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 0.1% Nonidet P-40® (NP-40), and protease inhibitor cocktail and homogenized by pestle homogenizer and 25-gauge needle. The lysates were centrifuged at 200 \( \times g \) for 1 min and the supernatant was collected. The supernatant was centrifuged at 750 \( \times g \), for 10 min and the pellet was resuspended with 50 mM Tris buffer containing 0.5 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 1% NP-40, and protease inhibitor cocktail for 60 min at 4°C with occasional mixing. The suspension was centrifuged at 12000 \( \times g \) for 20 min and the supernatant was collected as a plasma membrane fraction. For whole lysates, L6 myotubes or muscle tissues were lysed with 20 mM Tris buffer, pH 8.0, containing 300 mM NaCl, 1% sodium deoxycholate, 1 mM DTT, 0.2% Sodium dodecyl sulfate (SDS), 20 mM NaF, 2 mM Na₃VO₄, 2% NP-40, and protease inhibitor cocktail; and homogenized by pestle homogenizer and 25-gauge needle. The lysates were centrifuged at 12000 \( \times g \) for 20 min and the supernatant was collected as a whole fraction.

Western Blotting Analysis Proteins in myotubes and muscle tissues were separated with SDS-polyacrylamide gels and electrophoretically transferred to the polyvinylidene fluoride (PVDF) membranes. After blocking using Blocking-one™ solution (Nacalai Tesque, Kyoto, Japan), the membranes were washed with TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.06% Tween 20). The antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO CO., LTD., Osaka, Japan) and incubated overnight at 4°C. The membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized using ImmunoStar LD (Wako Pure Chemical Industries, Ltd.) and detected with Light-Capture II (ATTO Corp., Tokyo, Japan).

Animal Treatment Male ICR mice were purchased from SLC Japan, Inc. (Shizuoka, Japan) and maintained at 23 ± 2°C with a 12-h light-dark cycle. The mice were fed a pelleted diet

![Diagram](https://via.placeholder.com/150)
(Research Diets, Tokyo, Japan) for a week. All experimental procedures were in accordance with the guidelines for animal experiments of Kobe University Animal Experimentation Regulation with the permission of Kobe University Institutional Animal Care and Use Committee (Permission #1-27-05-09). These mice were subjected to an oral glucose tolerance test (OGTT) for the detection of GLUT4 translocation and its related signaling pathways. For OGTT, the mice aged 6 weeks were divided into five groups \((n = 4–5)\) with similar average weights (weight av. 16.5 g). After a 16h fast, 0.32, 3.2, 32 or 64 mg/kg/10 mL of EGC-M5 dissolved in saline was administered orally to mice with a gastric metal probe. Only saline (10 mL/kg) was administered to the vehicle control group. After 1 h of EGC-M5 administration, 1.0 g/kg/10 mL of glucose aqueous solution was orally administered to all mice. Blood samples were collected from the tail vein at 0 (before administration), 15, 30, 60 and 120 min after an oral dosage of glucose with heparinized tubes. Blood samples (25 µL) were centrifuged at 9600 × g for 10 min at 4°C. Resultant plasma \((2 \mu \text{L})\) was measured for glucose levels using Labassay1 Glucose Wako kit (Wako Pure Chemical Industries, Ltd.).

For measurement of GLUT4 translocation and its related signal pathways, mice aged 8 weeks were divided into four groups \((n = 5)\) with similar average weights (weight av. 41.8 g). After a 16h fast, 3.2 mg, 32 mg or 64 mg/kg/10 mL of EGC-M5 dissolved in saline was administered orally to mice with a gastric metal probe. Only saline (10 mL/kg) was administered to the vehicle control group. The mice were sacrificed 60 min after the administration of EGC-M5 under anesthesia using sodium pentobarbital and euthanized by exsanguination from cardiac puncture. The soleus muscle was collected from the hind legs, and its plasma membrane fraction and tissue lysate were prepared and subjected to Western blot analysis. At the same time, blood samples were collected from the cardiovascular organ. Blood samples were used for the quantitative analysis of metabolites of EGC-M5 as described below.

GLUT4 Translocation and Its Related Signal Pathways in Mice Plasma membrane fraction and tissue lysate of soleus muscle were prepared according to Nishiumi and Ashida's protocol.\(^{30}\) Proteins in the plasma membrane fraction and tissue lysate were separated by SDS-polyacrylamide gels and transferred to the PVDF membranes. After blocking, the membranes were washed and incubated with the specified primary antibodies overnight at 4°C, followed by incubation of the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized using ImmunoStar1 LD (Wako Pure Chemical Industries, Ltd.) and detected with Light-Capture II (ATTO Corp.).

Quantitative Analysis of Plasma Metabolites after Oral Administration of EGC-M5 We performed the quantitative analysis of plasma metabolites with the intent to confirm EGC-M5 had been absorbed at the time of the in vivo effects of EGC-M5 on GLUT4 translocation, phosphorylation of the signal pathways and OGTT in mice. Plasma samples were prepared by centrifuging the blood samples at 3000 g for 10 min at 4°C (MX-301, TOMY SEIKO CO., LTD., Tokyo, Japan). Aliquots (200 µL) of plasma samples were added to 0.2 M sodium acetate buffer (pH 4.0) and 1 mL of acetonitrile. The solution was mixed well, and 10000× g was centrifuged for 10 min at 10°C. The supernatant was placed in a new vessel, and the pellet was washed twice using 1 mL of 80% aqueous methanol. After centrifugation, the supernatant was collected and then evaporated to dryness with Centrifugal Concentrator CC-105 (TOMY SEIKO Co., LTD.). The residue obtained was dissolved in 200 µL of 5% aqueous methanol. The resultant solution was filtered with DISMIC-13HP (Toyo Roshi Kaisha Ltd., Tokyo, Japan) and subjected to LC-electrospray ionization (ESI)-MS\(^n\) and LC/MS/MS analysis.

The LC-ESI-MS\(^n\) analysis for the identification of plasma metabolites was performed using a Thermo Scientific\(^\text{TM}\) UltiMate\(^\text{TM}\) 3000 RSLCnano HPLC and an LQ Fleet\(^\text{TM}\) system (Thermo Fisher Scientific K. K., Yokohama, Japan). HPLC conditions for LC-ESI-MS\(^n\) analysis were according to the method described in our previous paper,\(^{13,14}\) essentially with the negative ion polarity mode set for the ESI source. Analysis was initially carried out using a full scan, with data-dependent MS2 and MS3 scanning from \(m/z\) 100 to 1000. Plasma metabolites including conjugates, were characterized and identified from precursor MS ion and MS\(^n\) fragmentation.\(^{31–33}\)

Glucuronide form of EGC-M5 (EGC-M5-GluUA) and sulfate form of EGC-M5 (EGC-M5-Sul) were prepared according to the previously reported method.\(^{35}\) LC/MS/MS analysis for quantification of plasma metabolites was conducted according to the procedure described in previous reports.\(^{31}\) The standard prepared solutions of EGC-M5-GluUA and EGC-M5-Sul were analyzed by the LC/MS/MS system, and the calibration curves were obtained by plotting the MS intensity of each reference standard against the concentration. Each metabolite was measured using the multiple-reaction monitoring (MRM) mode (Q1/Q3: EGC-M5: 207.10/145.10, EGC-M5-GluUA: 382.95/207.30, EGC-M5-Sul: 286.93/207.20).

Statistical Analysis All values are expressed as the mean ± standard error of the mean (SEM). Statistically significant differences between control and test substance-treated groups were performed using Dunnett’s test. The statistical calculations were done with IBM SPSS Statistics 19 (IBM Japan, Tokyo) (Figs. 2 and 6) or JMP\(^\text{®}\) (SAS Institute Inc., U.S.A.) (Figs. 3–5, 7, and 8). Differences are considered significant at \(p < 0.05\).

RESULTS AND DISCUSSION

Firstly, 11 kinds of EGCG metabolites, illustrated in Fig. 1, were examined for 2-DG uptake in rat skeletal muscle-derived L6 myotube cells treated with 3 µM of each metabolite for 15 min. Comparative potential of glucose uptake in L6 myotubes is shown in Fig. 2(A). It was found that EGC-M5, EGC-M6, EGC-M7, and EGC-M11 increased glucose uptake significantly. Glucose uptake enhancement of each metabolite was 164.2% (EGC-M5), 165.2% (EGC-M6), 167.6% (EGC-M7), and 146.3% (EGC-M11) compared with DMSO-treated negative control cells. These results indicate some EGCG metabolites have insulin-like activity. The effect of these metabolites was slightly lower than that of 100 nM insulin-treated cells (198.6%) but higher than that of 100 nM EGCG treated cells (132.1%). Structural relevance among these effective metabolites has not been found. Based on our previous research in rats, the main metabolite of EGCG and EGCG is considered to be EGC-M5.\(^{31,12}\) Thus in this study further research was conducted focusing on EGC-M5. When L6 myotubes were treated with EGC-M5 in concentrations...
ranging from 1 nM to 10 µM, the glucose uptake was found to be significantly promoted at the concentrations of 1, 3, and 10 µM, as shown in Fig. 2(B). This is the first report to our knowledge on the promotive effect of EGCG microbial metabolites on glucose uptake in muscle cells. This observation indicates that several EGCG metabolites could be bioactive molecules that improve insulin resistance in the body.

Next, we attempted to determine whether EGC-M5 could promote GLUT4 translocation from the intracellular pool to the plasma membrane in L6 skeletal muscle, using immunoblot analyses by Western blotting. As shown in Fig. 3, EGC-M5 could significantly promote GLUT4 expression in L6 myotube cells at concentrations of 1.0, or 3.0 µM. In particular, L6 myotubes treated with 3.0 µM of EGC-M5 exhibited this effect strongly, and to the same degree as 1 nM AICAR, which is an activator of AMPK, suggesting that EGC-M5 could effectively promote GLUT4 translocation in L6 skeletal muscle cell. In the case of intact EGCG, a significant promotive effect on glucose uptake ability accompanied by GLUT4 translocation was detected at 1 nM, indicating that the in vitro effect of EGC-M5 for glucose uptake ability accompanied by GLUT4 translocation is lower than that of EGCG.

GLUT4 translocation is known to be induced by phosphorylation of signaling proteins through both insulin-dependent and insulin-independent pathways. Thus, phosphorylation of protein kinase B (Akt), protein kinase C (aPKC) and PI3K was examined as an insulin-dependent signaling pathway and that of AMPK was examined as insulin-independent pathway by Western blot analysis. It was found that phosphorylation of AMPK was significantly increased in L6 myotube cells treated with 1.0 and 3.0 µM of EGC-M5. In particular, the 3.0 µM EGC-M5-treated cells showed a comparatively intense level of phosphorylation and to the same degree as 1 nM of the positive control AICAR (Fig. 4). This result indicates that EGC-M5 could promote GLUT4 translocation via activation of insulin-independent AMPK signaling pathway. On the other hand, there was no significant difference in p-PI3K/PI3K, p-Akt/Akt (Ser473), p-Akt/Akt (Thr308) and p-aPKC/aPKC as the insulin-dependent signaling proteins in EGC-M5-treated L6 myotube cells under our experimental conditions (Fig. 5). These findings demonstrated that EGC-M5 could promote...
translocation of GLUT4 to the plasma membrane by activating AMPK, leading to an increased glucose uptake into skeletal muscles.

It has been reported that activation of AMPK by adiponectin could promote glucose metabolism through the stimulation of glucose uptake in skeletal muscle and also fatty-acid oxidation. Furthermore, the activation of AMPK has been studied with regard to an inhibition mechanism in cancer cell growth. Therefore, AMPK activators are targets of investigation because of their applications in the treatment and prevention of metabolic syndrome, hypertension, hyperglycemia and hyperlipidemia, and prevention of cancer. Procyanidin, which is a plant polyphenol contained in foods such as nuts, berries, cacao and red wine, has been reported to be an AMPK activator. Cacao liquor procyanidin (CLPr) extract has been reported to promote phosphorylation of AMPK and translocation of GLUT4 in the plasma membrane of skeletal muscle. Investigation into AMPK activators in dietary polyphenols, including metabolites such as EGC-M5, is a subject of considerable interest for prevention of metabolic syndrome as well as cancer prevention.

As mentioned above, it is newly discovered in this study that EGC-M5 has a promotive effect on glucose uptake, GLUT4 translocation, and AMPK phosphorylation in skeletal muscle cells. From these results, it can be deduced that EGC-M5 may have the ability to improve glucose tolerance through enhancement of glucose uptake in skeletal muscle.

![Fig. 4. Effect of EGC-M5 on Phosphorylation of AMPK in L6 Myotubes](image)

L6 myotubes were treated with EGC-M5 at 0.3, 1.0 or 3.0 µM for 15 min. DMSO and 1 nM AICAR was used as the negative and positive controls, respectively. The protein of p-AMPK and AMPK in the cell lysate was detected by Western blot analysis and the density of each band was analyzed and normalized. The results are presented as the mean ± SEM (n = 3). Asterisks indicate significantly different from the control group (*p < 0.05, Dunnett’s test).

![Fig. 5. Effect of EGC-M5 on Phosphorylation on the Insulin-Signaling Pathway in L6 Myotubes](image)

L6 myotubes were treated with EGC-M5 at 0.3, 1.0 or 3.0 µM for 15 min. DMSO and 100 nM insulin were used as the negative and positive controls, respectively. Immunoblotting analysis to determine (A) p-PI3K and PI3K; and (B) p-Akt serine 473 and Akt; (C) p-Akt threonine 308 and Akt; (D) p-aPKC and aPKC were performed by Western blot analysis and the density of each band was analyzed and normalized. The results are presented as the mean ± SEM (n = 3). Asterisks indicate significant differences from the control group (*p < 0.05, Dunnett’s test).
Based on this assumption we next conducted an in vivo experiment to investigate this potential. Evaluation of EGC-M5 function in vivo was performed by oral glucose tolerance test (OGTT) which is commonly used in human diagnostic modalities for evaluation of insulin resistance and diabetes mellitus. In addition to measurement of plasma glucose level by OGTT, measurement of GLUT4 translocation and its related signal pathways in soleus muscle after oral dosage of EGC-M5 were analyzed by Western blotting.

Figure 6(A) shows the effect of single oral administration of EGC-M5 on the plasma glucose response to an oral load of glucose. In the saline control group, plasma glucose level was increased in response to oral glucose loading and reached a maximum value after 15 min (216.3 ± 21.5 mg/dL), decreased after 30 min (171.5 ± 27.0 mg/dL), and reached the normal value level by 120 min. In the EGC-M5 (32 mg/kg of body weight) treated group, postprandial hyperglycemia was significantly suppressed compared with the saline control group at 15 min (150.5 ± 13.6 mg/dL) and 30 min (108.5 ± 17.2 mg/dL) after oral glucose loading. The area under the curve of the plasma glucose levels (AUC) calculated at 0–120 min in the OGTT is shown in Fig. 6(B). A significant decrease in AUC was found in the 32 mg/kg body weight treated group, while in the 64 mg/kg body weight EGC-M5 treated group, significant suppression of blood glucose level elevation was observed at only 15 min following the glucose load in the OGTT test (Fig. 6(A)). However a significant decrease of the AUC value was not detected in the 64 mg/kg of body weight group as shown in Fig. 6(B). It was determined that suppression of the increase in postprandial plasma glucose levels by EGC-M5 is remarkably effective in the 32 mg/kg of body weight treated group but not the 64 mg/kg of body weight treated group. From these observations, it is suggested there is likely to be an optimal dose for the improvement effect on glucose tolerance by EGC-M5 in vivo.

Furthermore, we investigated in vivo expression of GLUT4 on the plasma membrane and phosphorylation of PI3K, Akt, and AMPK in soleus muscle of ICR mice after single oral administration of EGC-M5. Dosages of EGC-M5 to mice were adjusted to 3.2, 32 and 64 mg/kg of body weight, the same dosages as in the OGTT test.

The results of the analysis by Western blotting are shown as an expression of GLUT4 (Fig. 7), and phosphorylation of signal pathways (Fig. 8). Regarding the translocation of GLUT4 in the soleus muscle of ICR mice treated with EGC-M5, it can be stated that only the 32 mg/kg body weight treated group significantly increased GLUT4 translocation in the plasma membrane as compared with results of the saline dosed control group (Fig. 7). In the 64 mg/kg dosed body weight group, a significant increase of GLUT4 translocation was not detected. None of the concentrations affected the expression level of GLUT4 in the tissue lysate. The dose at which significant
Glucose tolerance in vivo by enhancement of glucose uptake into muscle tissue. The phosphorylation of signal proteins in rats administrated EGC-M5 at concentrations 3.2, 32 or 64 mg/kg of body weight are provided in Fig. 8. There was no significant increase in the phosphorylation level of p-PI3K/PI3K (Fig. 8(A)), p-Akt/Akt (Ser473) (data not shown) and p-Akt/Akt (Thr308) (data not shown). These results agree with in vitro examination of L6 myotubes. However a significant increase in the phosphorylation level of p-AMPK/AMPK was observed in both the 32 and 64 mg/kg body weight EGC-M5 dosed groups (Fig. 8(B)). The finding that EGC-M5 activates AMPK potential in skeletal muscle tissue is consistent with in vitro examination of L6 myotubes. Concerning phosphorylation of AMPK, significant AMPK activation was promoted in both the 32 and 64 mg/kg of body weight treated groups. This result which showed a significant difference in not only the 32 mg/kg but also the 64 mg/kg of body weight dose group is not in agreement with results of OGTT and GLUT4 translocation in vivo.

From the Figs. 6, 7 and 8, it can be stated that EGC-M5 has the potential to contribute to improvement of postprandial hyperglycemia and insulin resistance in vivo. We analyzed plasma metabolites at 60 min after oral administration of EGC-M5 with LC-ESI-MS and LC/MS/MS analysis to confirm absorption in the body. From the LC-ESI-MS analysis, EGC-M5 aglycone, EGC-M5 monoglucuronide (EGC-M5-GlcUA) and EGC-M5 monosulfate (EGC-M5-Sul) were detected as plasma metabolites after oral administration of EGC-M5. The concentrations of EGC-M5 metabolites detected in the plasma by LC/MS/MS analysis are listed in Table 1. The EGC-M5 aglycone was detected at the concentrations of 0 µmol/mL (3.2 mg/kg dosed), 5.62 µmol/mL (32 mg/kg dosed) and 3.15 µmol/mL (64 mg/kg dosed) in plasma samples 60 min after oral dosage of EGC-M5. As shown in Figs. 6(B) and 7(A), significant lowering of blood glucose AUC and significant promotion of GLUT4 translocation in the soleus muscle were observed in only the 32 mg/kg EGC-M5 dosed group and not in the 64 mg/kg dosed group. Regarding plasma concentration of EGC-M5 aglycone, in the 32 mg/kg dosed group this was found to reach 5.62 µmol/mL which is higher than that of the 64 mg/kg dosed group, and thus it is suggested that in the 32 mg/kg dosed mice group, EGC-M5 could enter into muscle tissue and still retain an effective concentration. On the basis of these results, we reached the tentative conclusion that the EGC-M5 aglycone absorbed in the blood may be contributing to improvement of glucose tolerance in mice.

Additionally, it was found that most of the EGC-M5 was the conjugated form of glucuronide and sulfate in the blood after oral administration. From these observations, it is regarded to be likely that the glucuronide and sulfate conjugation takes place during the absorption process through the digestive tract. Further work is needed to investigate the relevance of both conjugates, EGC-M5-GlcUA and EGC-M5-Sul in the prevention of hyperglycemia.

We previously examined the effects of EGCG metabolites on angiotensin-converting enzyme (ACE) inhibitory activity in vitro and blood pressure in spontaneously hypertensive rats. It was found that EGC-M5 and EGC-M7 had an inhibitive effect on ACE enzyme in vitro and hypotensive effects in vivo. Kiryu et al. reported that ACE inhibitors sig-

![Fig. 8. Effect of EGC-M5 on Phosphorylation of PI3K and AMPK in Skeletal Muscle of Mice](image)

ICR mice were treated with EGC-M5 at 3.2, 32 or 64 mg/kg body weight or saline as a control. Tissue lysate of skeletal muscle was prepared 60 min after the administration and subjected to Western blot analysis to determine (A) p-PI3K and PI3K (B) p-AMPK and AMPK and the density of each band was analyzed and normalized. The results are presented as the mean ± SEM (n = 5). Asterisks indicate significant differences from the control group (*p < 0.05, Dunnett’s test).

Table 1. Quantitation of Plasma Metabolites after 60 min of EGC-M5 Oral Dosage in Mice

| Plasma metabolites (µmol/mL) | 3.2 mg/kg of dosage | 32 mg/kg of dosage | 64 mg/kg of dosage |
|------------------------------|---------------------|-------------------|-------------------|
| EGC-M5 aglycone              | 0                   | 5.62 ± 1.31       | 3.15 ± 2.27       |
| EGC-M5-GlcUA                 | 1.56 ± 0.73         | 38.5 ± 4.63       | 57.45 ± 8.91      |
| EGC-M5-Sul                   | 2.62 ± 1.47         | 24.3 ± 3.31       | 38.30 ± 12.97     |

Data are expressed as the mean ± SEM (n = 5).
nificantly decreased cholesterol, triglyceride and blood sugar levels in hypertensive patients with slight hyperlipidemia or diabetes mellitus. It was suggested that ACE inhibitors play a useful role in metabolizing lipid and glucose in serum, as well as being antihypertensive agents. Given the relationship between inhibition activity of ACE enzyme and hypoglycemic effect, it is considered that the improvement of postprandial hyperglycemia by EGC-M5 could involve both promotion of glucose uptake into skeletal muscle tissue and ACE inhibition action.

In addition, AMPK has been considered to be an important therapeutic target for controlling human diseases including metabolic syndrome and cancer as described above, and thus there is a strong possibility EGC-M5 may contribute systemically through AMPK activation. These findings indicate that metabolites play a crucial role in metabolic regulation in the body following green tea intake.

Yashi et al. reported that EGCG administration reversed insulin resistance and neuroinflammation in the brain of C57BL/6J mice fed a long-term high-fat and high-fructose diet (HFFD). Recently, Unno et al. discovered that EGC-M5 could significantly prolong neurite length of human neuroblastoma SH-SY5Y cells, and increase the number of these cells significantly. Since EGC-M5 is the major metabolite of EGCG in the body, it is possible that EGC-M5 could also contribute to the amelioration of cognitive disorder in vivo.

Reports on the functions of catechin metabolites have been increasing in recent years, however information is still lacking on their mechanisms involved in beneficial health effects. We expect these advances in research into functionality of EGCG metabolites will lead to more in-depth clarification of the mechanisms of physiological functions following EGCG intake.

This is the first report to our knowledge on the antiadipogenic effects of EGCG microbial metabolites. As the major metabolite, EGC-M5 was found to have the potential to improve glucose tolerance through promotion of GLUT4 translocation to the plasma membrane accompanied by phosphorylation of AMPK in skeletal muscle. Considering evidence of the high bioavailability of the microbial metabolites after oral dosage of EGCG, it is likely that EGC-M5 is contributing to improvement of glycemic control following the consumption of green tea. As a next step, it would be useful to investigate the effect of EGC-M5 in the alleviation of chronic hyperglycemia with disease-model animals. Further studies are needed to clarify the contribution of microbial metabolites, including conjugates, on the various beneficial functions and effects following green tea consumption.

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