**Original paper**

**Black Tea Polyphenols Promotes GLUT4 Translocation through Both PI3K- and AMPK-dependent Pathways in Skeletal Muscle Cells**

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We previously reported that the intake of black tea promotes translocation of the insulin-sensitive glucose transporter (GLUT) 4 in skeletal muscle. In this study, we investigated whether black tea polyphenols (BTP) promote GLUT4 translocation in L6 myotubes. BTP promoted glucose uptake accompanied by GLUT4 translocation in L6 myotubes. As the molecular mechanism, BTP induced the phosphorylation of insulin receptor substrate-1, atypical protein kinase C, Akt Thr308, Akt substrate 160, and AMP-activated protein kinase (AMPK), but did not affect that of Akt Ser473. BTP increased glycogen accumulation through inactivation of glycogen synthase kinase 3β (GSK-3β). Theaflavin, one of the major components in black tea, also promoted the glucose uptake accompanied by GLUT4 translocation observed with BTP in L6 myotubes. These results indicate that BTP activates both PI3K- and AMPK-dependent pathways to promote GLUT4 translocation and glycogen accumulation in skeletal muscle cells. Moreover, theaflavin is one of the active components in BTP.

Keywords: black tea polyphenols, glucose transporter, AMP-activated protein kinase, skeletal muscle

**Introduction**

Glucose transporters (GLUTs) play an important role in the regulation of blood glucose levels. GLUT4 is specifically expressed in skeletal muscle and adipose tissue, where it is mainly localized in intracellular storage vesicles. GLUT4 translocates to the plasma membrane by various stimuli and takes up glucose to reduce postprandial hyperglycemia (Belman et al., 2014). In skeletal muscle, the translocation is regulated by insulin and AMP-activated protein kinase (AMPK) signaling pathways (Sheena et al., 2011). Binding of insulin activates the tyrosine kinase activity of its receptor, which phosphorylates insulin receptor substrate-1 (IRS-1), followed by phosphorylation of the p85 regulatory subunit of phosphatidylinositol 3'-kinase (PI3K). Activated PI3K induces phosphorylation of Akt and atypical protein kinase C (aPKC) in turn to promote GLUT4 translocation. AS160 (Akt substrate of 160 kDa), which has a Rab GTPase-activating domain, is a direct substrate of Akt, and plays an important role in the regulation of GLUT4 trafficking (Ramm et al., 2006). These reports indicate that GLUT4 translocation is regulated by a complex cascade of multiple protein kinases, and GLUT4 finally appears on the plasma membrane and incorporates large amounts of glucose.

In skeletal muscle, insulin promotes glycogen accumulation, which contributes to the prevention of postprandial hyperglycemia (Manchester et al., 1996). Defects in glucose uptake and glycogen synthesis, which occur in diabetes, are implicated in the development of hyperglycemia and other complications (Defronzo et al., 1992). It is reported that defects in insulin-stimulated glycogen synthesis in the liver and muscle are major factors in the...
onset of postprandial hyperglycemia in patients with type 2 diabetes (Liljioja et al., 1986). Insulin enhances the incorporation of glucose into glycogen by activating glycogen synthase (GS) through its dephosphorylation (Roach, 2002). Activation of GS by insulin leads to phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β) as a direct substrate of Akt (Cross et al., 1995).

Black tea, a fully fermented type of tea, characteristically contains theaflavins and thearubigins, which are formed by the oxidation and polymerization of catechins in tea leaves during fermentation. Theaflavins, namely theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate, possess a benzotropolone skeleton that is formed from the co-oxidation of selected pairs of catechins. In black tea leaves, the composition of catechins, theaflavins and thearubigins is 3–10%, 2–6%, and >20%, respectively, in the water-extractable material by dry weight (Balentine et al., 1997, Yang et al., 2008, Haslam, 2003). It was reported that black tea and tea polyphenols, in particular catechins, have various beneficial effects at the physiological and cellular levels, such as anti-hyperglycemic and anti-obesity effects (Nishiumi et al., 2010, Lin et al., 2006, Wolfram et al., 2006, Yamashita et al., 2012, 2014). Recently, many researchers have focused their attention not only on catechins but also theaflavins due to their antioxidant, anti-inflammatory, and anti-tumor effects (Kumar et al., 2010, Sharma and Rao, 2009).

We previously reported that black tea improved postprandial hyperglycemia by promoting GLUT4 translocation to the plasma membrane in skeletal muscle (Yamashita et al., 2012). However, the underlying molecular mechanism by which black tea promotes GLUT4 translocation in skeletal muscle is not yet fully understood. Therefore, in this study we aimed to clarify this issue by investigating the effects of black tea polyphenols (BTP) and theaflavin in L6 cells.

Materials and Methods

Chemicals and antibodies  BTP was kindly provided by ITO EN, Ltd. (Makinoara, Japan). The composition of polyphenols in BTP is shown in Table 1. Theaflavin was purchased from Nagara Science Co., Ltd. (Gifu, Japan). For the glucose uptake assay, [1, 2-3H]-2-deoxy-D-glucose (2-DG) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). For western blotting, anti-GLUT4 goat IgG, anti-IRβ rabbit IgG, anti-phospho-PIK3K goat IgG, anti-phospho-Akt substrate (Thr308) rabbit IgG, anti-Akt rabbit IgG, anti-phospho-Akt substrate (119B7E) rabbit IgG, anti-AMPKα rabbit IgG, anti-PKCa rabbit IgG, anti-phospho-AMPK-α (Thr 172) rabbit IgG, anti-phospho-PKCα/β (Thr 410/403) rabbit IgG and anti-phospho-GSK3α/β (Ser21/9) rabbit IgG antibodies were from Cell Signaling Technology Co. (Danvers, MA, USA). All other reagents employed were of the highest grade available commercially.

Cell culture and treatments  L6 cells were maintained in modified Eagle’s medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C in a 5% CO2 atmosphere. When L6 cells reached confluence, the medium was replaced every 2 days with differentiation medium consisting of MEM supplemented with 2% FBS. Fully differentiated myotubes (8 days after differentiation) were used for the following experiments. After the cells were serum-starved for 16 h in MEM containing 0.2% BSA, the cells were incubated with the indicated concentration of BTP or theaflavin for 15 min and then immediately washed twice with ice-cold Krebs-Ringer phosphate-HEPES buffer (KRH; 50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM CaCl2, and 1.3 mM MgSO4). Cells were treated with 100 nM insulin for 15 min or with 1 mM AICAR for 2 h as a positive control.

Western blot analysis  We prepared plasma membrane fractions and cell lysates from L6 cells as previously described (Nishiumi and Ashida, 2007). The plasma membrane fraction was used to detect GLUT4 translocation and IRβ expression. Cell lysates were used to detect the expression and phosphorylation of proteins related to GLUT4 translocation. Primary and secondary antibodies were diluted 1:10,000 and 1:20,000 ~ 1:200,000, respectively, in Can Get Signal (Toyobo Co., Ltd., Osaka, Japan).

<table>
<thead>
<tr>
<th>Table 1. Composition of polyphenols in BTP</th>
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<tr>
<td>Component</td>
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<tr>
<td>Epigallocatechin gallate</td>
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<tr>
<td>Epicatechin gallate</td>
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<tr>
<td>Galloatechin gallate</td>
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<tr>
<td>Catechin gallate</td>
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<td>Epigallo catechin</td>
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<td>Epicatechin</td>
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<tr>
<td>Galloatechin</td>
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<td>Catechin</td>
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<td>Total catechins</td>
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<tr>
<td>Theaflavin</td>
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<tr>
<td>Theaflavin-3-gallate</td>
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<tr>
<td>Theaflavin-3′-gallate</td>
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<tr>
<td>Theaflavin-3,3′-digallate</td>
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<tr>
<td>Total theaflavins</td>
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<tr>
<td>Total polyphenols</td>
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<tr>
<td>Caffeine</td>
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Protein bands were visualized using ImmunoStar LD (Wako Pure Chemical Industries) and detected with a light-Capture II (Atto Corp., Tokyo, Japan). The density of specific bands was determined using ImageJ image analysis software provided by the National Institutes of Health (NIH, Bethesda, MD, USA).

**Glucose uptake assay**  L6 cells were differentiated and serum-starved in a 24-well plate as described above. The cells were incubated for 15 min with the indicated concentration of BTP or theaflavin in KRH containing 0.1% (w/v) BSA. Then, 6.5 mM (0.5 µCi) [3H]-2-DG was added to the cells and incubated for 5 min at 37°C. As the positive and negative controls, cells were treated with 100 nM insulin and DMSO (final concentration of 0.1%) for 15 min, respectively. The glucose uptake was terminated by immediate washing of the cells four times with ice-cold KRH, and the cells were solubilized with 0.05 N NaOH. Nonspecific uptake was measured in the presence of 20 µM cytochalasin B as an inhibitor of glucose transporters. The radioactivity in the solubilized solution was measured with liquid scintillation cocktail (Ultima Gold XR; Perkin Elmer, Boston, MA, USA) using a liquid scintillation counter (LSC6101B; Hitachi Aloka Medical, Tokyo, Japan).

**Measurement of glycogen level**  Serum-starved L6 cells in a 6-well plate were incubated with the indicated concentration of BTP for 15 min. The cells were immediately washed twice with ice-cold KRH and dried at 105°C for 10 min. As the positive and negative controls, cells were treated with 100 nM insulin and DMSO (final concentration of 0.1%) for 15 min, respectively. The dried cells were suspended in 200 µL H2O, and the amount of glycogen in the suspension was measured by a commercial kit (Biovision, Milpitas, CA, USA).

**Statistical analysis**  Statistical analyses were performed with Dunnett’s multiple comparison test using EXCEL-Toukei Ver. 7.0. The level of significance was defined as $p < 0.05$.

**Results**

Effects of BTP on GLUT4 translocation to the plasma membrane and glucose uptake in L6 cells  Previous studies have shown that black tea possesses anti-hyperglycemic effects (Nishiumi et al., 2010, Lin et al., 2006). Moreover, our previous study demonstrated that black tea improved postprandial hyperglycemia by promoting GLUT4 translocation to the plasma membrane in mouse skeletal muscle (Yamashita et al., 2012). Therefore, in this study, we investigated the molecular mechanism of BTP, which contains abundant theaflavins, on GLUT4 translocation to the plasma membrane and glucose uptake in L6 cells. First, we determined the effective concentration of BTP for GLUT4 translocation. As shown in Fig. 1A, BTP promoted GLUT4 translocation compared with DMSO-treated cells, and 0.1 µg/mL BTP had almost the same effect as 100 nM insulin. Significant translocation was observed at 1 µg/mL BTP. Under the same experimental conditions, BTP also promoted glucose uptake in L6 cells (Fig 1B), with the highest activity observed at 1 µg/mL. BTP used in this study did not show any cytotoxicity under our experimental conditions (data not shown). These results indicate that BTP increases glucose uptake and promotes GLUT4 translocation to the plasma membrane in L6 cells.

**BTP promotes GLUT4 translocation to the plasma membrane through both insulin and AMPK signaling pathways**  To clarify the mechanism by which BTP promotes GLUT4 translocation in L6 cells, we examined the phosphorylation status of several
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Fig. 3. Effect of BTP on glycogen accumulation and activation of glycogen synthase kinase in L6 cells. Differentiated L6 cells were incubated with BTP at the indicated concentrations or 100 nM insulin as a positive control for 15 min. (A) Glycogen content was measured using a commercially available kit. (B) Phosphorylation and expression of GSK3β were detected by western blotting.

proteins in the insulin and AMPK signaling pathways. As shown in Fig. 2A, BTP induced the phosphorylation of IRS-1, PI3K, PKCζ/ζ, Akt on Thr308 and AS160, as observed for insulin. However, BTP did not increase the phosphorylation of Akt on Ser 473, whereas insulin phosphorylated Akt at both residues. Moreover, BTP induced the phosphorylation of AMPK as well as AICAR, a specific activator of AMPK (Fig. 2B). Neither BTP nor insulin affected the expression levels of these proteins (Fig. 2). These results indicate that BTP promotes GLUT4 translocation in L6 cells through both PI3K- and AMPK-dependent pathways.

**BTP enhances glycogen accumulation by inactivating glycogen synthase kinase** To clarify glucose utilization, which was incorporated by the action of BTP, in L6 skeletal muscle cells, we investigated the effects of BTP on glycogen synthesis. As shown in Fig. 3A, BTP significantly increased glycogen accumulation in L6 cells, as observed for insulin. The highest accumulation was observed at 1 μg/mL BTP. In skeletal muscle, insulin induces glycogen synthesis by phosphorylating GSK3, which is located downstream of Akt (Cross et al., 1995). Therefore, we investigated whether BTP promotes the phosphorylation of GSK3β in L6 cells (Fig. 3B). BTP promoted phosphorylation in a dose-dependent manner; significant phosphorylation was observed at 1 μg/mL BTP. BTP treatment similar to the insulin treatment compared with DMSO-treated cells, although 10 μg/mL BTP down-regulated the phosphorylation. These results suggest that BTP enhances glycogen accumulation through inactivating GSK3β.

**Effects of theaflavin on glucose uptake and GLUT4 translocation to the plasma membrane in L6 cells.** Theaflavin is a typical polyphenol found in black tea. We investigated whether theaflavin promotes GLUT4 translocation to the plasma membrane and glucose uptake in L6 cells. The level of GLUT4 translocation to the plasma membrane by 100 nM theaflavin was almost the same as that by 1 μg/mL BTP (Fig. 4A). As shown in Fig. 4B, theaflavin increased glucose uptake in a dose-dependent manner, and a significant increase was observed at 100 nM in L6 cells. From the composition of BTP (Table 1), 1 μg/mL BTP contains approximately 80 nM theaflavin. Therefore, theaflavin will contribute to BTP-induced GLUT4 translocation to the plasma membrane.

**Discussion**

Several natural products including tea catechins have the potential to prevent and treat hyperglycemia and diabetes mellitus. We previously reported that black tea improves postprandial hyperglycemia by promoting GLUT4 translocation to the plasma membrane in vivo (Yamashita et al., 2012). In this study, we found that BTP promoted GLUT4 translocation and glucose uptake through PI3K- and AMPK-dependent pathways in L6 cells (Figs. 1 and 2). Moreover, we found that BTP induced the activation of AS160, located downstream of Akt, without affecting the phosphorylation of Akt Ser473. This is a unique mechanism for regulating GLUT4 translocation. Akt plays an important role in regulating glucose metabolism, and the phosphorylation of Akt at both amino acid residues, Ser473 and Thr308, leads to the full activation of this kinase (Vanhaesebroeck and Alessi, 2000). It is reported that Thr308 was phosphorylated by phosphoinositide-dependent kinase-1 (PKD1) while Ser473 was phosphorylated by mammalian target of rapamycin (mTOR)/Rictor complex (Kumar et al., 2007). This report suggests that full activation of Akt is necessary for glucose uptake activity in skeletal muscle. On the other hand, ErbB3, a member of the EGF receptor family of tyrosine kinases, promoted GLUT4 translocation to the plasma membrane in L6 cells through the activation of PI3K and PKCζ/ζ without activating Akt (Cantó et al., 2004). From these results, we suggest that full activation of Akt is not necessary for BTP-induced GLUT4 translocation in skeletal muscle cells.

In this study, we also found that BTP promoted glycogen
accumulation in L6 cell (Fig. 3A). Our results coincide with those previously reported (Cross et al., 1995), which demonstrated that activated Akt induced the phosphorylation of GSK3β. GSK3 is a rate-limiting enzyme and causes the inactivation of GS for reduction of glycogen synthesis (Oreña et al., 2000, Henriksen and Dokken, 2006).

Tea abundantly contains polyphenols such as catechins and theaflavins. These compounds contribute to the various health-promoting effects of tea. BTP contained theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-digallate. As to the bioavailability of theaflavins, theaflavin and theaflavin-3-gallate were detected in the liver as free form at 0.5 and 0.05 nmol/g tissue and were also detected in the prostate as both free and conjugated forms, though these compounds were not detected in the plasma of mice (Henning et al., 2006). In a human study, the maximum theaflavin plasma concentrations of females and males were 1.8 and 0.9 nM, respectively, and the maximum concentrations in urine were 1.1 and 7.4 nM, respectively, 2 h after intake of 700 mg (approximately 1 mmol) mixed theaflavins (Mulder et al., 2001). These reports indicate that plasma levels of theaflavins are low, but certain amounts of theaflavins exist in the tissues as free form. In this study, we investigated theaflavin and found that theaflavin promoted both GLUT4 translocation to the plasma membrane and glucose uptake in L6 cells (Fig. 4). However, the concentrations employed were higher than physiological concentrations, suggesting that GLUT4 translocation observed in the in vivo study (Yamashita et al., 2012) was possibly due to the indirect effect of theaflavins, such as an incretin effect. Further studies are needed to investigate the detection of theaflavins in skeletal muscle and the molecular mechanism of GLUT4 translocation in animal studies, in particular, the incretin effect of theaflavins is interesting issue to be clarified.

In conclusion, BTP promoted GLUT4 translocation to the plasma membrane through both PI3K- and AMPK-dependent pathways in skeletal muscle cells. A proposed model for BTP-induced GLUT4 translocation and glycogen accumulation in skeletal muscle cells is shown in Fig. 5. Moreover, theaflavin also promoted both the GLUT4 translocation and glucose uptake in L6 cells. These results indicate that BTP may modulate blood glucose levels by its insulinomimetic action. Further, theaflavin is one of the active components in BTP.

References


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Fig. 5. A proposed mechanism of BTP-induced GLUT4 translocation and glycogen accumulation.

IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3′-kinase; PKC, protein kinase C; AS160, Akt substrate of 160 kDa; AMPK, AMP-activated protein kinase; GSK3β, glycogen synthase kinase 3β; GS, glycogen synthase.


