

# Comparison of Anti-Hyperglycemic Activities Between Low- and High-Degree of Polymerization Procyanidin Fractions from Cacao Liquor Extract

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## ABSTRACT

Procyanidins are thought to be effective for prevention of hyperglycemia. To clarify the degree of polymerization (DP) of procyanidins on anti-hyperglycemic effects, we prepared low-DP ( $DP \leq 3$ ) and high-DP ( $DP \geq 4$ ) procyanidin fractions from cacao liquor, and examined whether these procyanidin fractions prevent hyperglycemia *in vitro*. We found that both fractions promoted glucose uptake in L6 myotubes in a dose-dependent manner accompanied by translocation of GLUT4 to the plasma membrane. Moreover, both fractions stimulate phosphorylation of AMP-activated protein kinase (AMPK). In these beneficial effects, low-DP fraction was more effective than high-DP one. On the other hand, high-DP fraction showed greater inhibitory effect of intestinal  $\alpha$ -glucosidase activity than low-DP fraction in a cell free-incubation system. In conclusion, cacao liquor procyanidins have anti-hyperglycemic activities and low-DP procyanidins mainly contribute to stimulate glucose uptake by glucose transporter 4 translocation through AMPK-dependent pathway in skeletal muscle while high-DP procyanidins mainly contribute to inhibit the  $\alpha$ -glucosidase activity in small intestine.

Key words: cacao liquor procyanidin, hyperglycemia, GLUT4 translocation,  $\alpha$ -glucosidase, AMP-activated protein kinase

## INTRODUCTION

Type 2 diabetes mellitus is a serious health problem in many countries worldwide. The pathogenesis of type 2 diabetes mellitus involves progressive development of insulin resistance in peripheral tissues. Insulin resistance is associated with increased risk of cardiovascular diseases and diabetes<sup>(1)</sup>. Thus, prevention of an excess postprandial rise of blood glucose levels and improvement of insulin resistance are effective for management of diabetes mellitus.

Natural compounds with insulin mimetic activity have been proposed as a candidate for potential therapeutic agents in the prevention and/or treatment of metabolic syndrome and diabetes<sup>(2)</sup>. It is known that certain polyphenols have a potency to normalize blood glucose levels by inhibiting carbohydrate digestive enzymes including  $\alpha$ -glucosidase. Recently, insulin-sensitive glucose transporter 4 (GLUT4) is considered to be a novel target of polyphenols for prevention of hyperglycemia<sup>(3)</sup>. GLUT4 is expressed in adipose tissue and skeletal and cardiac muscles. Of these, skeletal muscle is the most important therapeutic target for hyperglycemia, because skeletal muscle accounts for approximately 80% of insulin-stimulated glucose uptake in the postprandial state<sup>(3)</sup>. The two main molecules that regulate

GLUT4-dependent glucose transport into the cytoplasm are phosphatidylinositol-3 kinase (PI3K) and AMP-activated protein kinase (AMPK). Several studies indicated that polyphenols have potential to increase translocation of GLUT4 through these signaling pathways in peripheral tissues, including skeletal muscle<sup>(2)</sup>.

Procyanidins, the oligomers and polymers of flavan-3-ols and consist of epicatechin and catechin subunits, are abundant in grapes, cocoa, and apples<sup>(4)</sup>. Recent reports indicated that monomers, dimers and trimers are absorbed into the body<sup>(5)</sup>. The evidences are being accumulated that that procyanidins possess various beneficial effects for health promotion including the prevention of diabetes mellitus. For example, grape seed procyanidin extract suppressed hyperglycemia in type 1 diabetic rats<sup>(6)</sup>, and black soybean seed extracts containing rich procyanidins also suppressed hyperglycemia and obesity in high-fat diet-fed mice<sup>(7)</sup>. Cacao liquor extract is rich in procyanidins, and it was also reported that intake of the extract prevented elevation of blood glucose level in diabetic obese mice<sup>(8)</sup>. However, the underlying molecular mechanisms by which procyanidins suppress hyperglycemia are not fully understood yet. In this study, we investigated that the effects of the low and high degree of polymerization (DP) procyanidin fractions from cacao liquor on glucose uptake activity, GLUT4 translocation, phosphorylation of AMPK, and inhibition of  $\alpha$ -glucosidase

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as the anti-hyperglycemic activities.

## MATERIALS AND METHODS

### I. Cacao Liquor Procyanidins

Cacao liquor procyanidin extract (CLPr) was prepared from cacao liquor<sup>(9)</sup>. The total amount of polyphenols in CLPr was 69.8%. Each polyphenol in CLPr was quantified by HPLC and liquid chromatography-mass spectrometry<sup>(9,10)</sup>. The amounts of individual procyanidins are represented as epicatechin equivalents. CLPr consisted of 4.28% of catechin, 6.12% of epicatechin, 3.60% of procyanidin B2, 0.75% of procyanidin B5, 2.28% of procyanidin C1 and 1.01% of cinnamtannin A2. Low-DP (DP ≤ 3) and high-DP (DP ≥ 4) procyanidin fractions were separated from CLPr as previously reported<sup>(11)</sup>. Their total polyphenol contents are 75.4 and 66.1%, respectively. Procyanidin composition in low-DP fraction was 10.03% catechin, 14.56% epicatechin, 8.43% procyanidin B2, 1.79% procyanidin B5, 5.25% procyanidin C1 and 0.23% of cinnamtannin A2, while that in high-DP fraction was 0.25% catechin, 0.39% epicatechin, 0.24% procyanidin B2, 0.31% procyanidin C1, 2.91% cinnamtannin A2, and other oligomeric and polymeric procyanidins.

### II. Cell Culture and Treatment with Cacao Liquor Procyanidins

Differentiation of L6 myotubes to myoblasts was carried out according to the previous report<sup>(12)</sup> with slight modification. Briefly, the cells on 24-well plates or 60-mm dishes with a semi-confluent state were cultured with DMEM containing 2% horse serum for 5 days. Myotubes were incubated in serum-free DMEM containing 0.2% w/v bovine serum albumin for 18 h, and then treated with CLPr, low- and high-DP fractions, or dimethylsulfoxide (DMSO) as a vehicle control. CLPr, high- and low-DP fractions (250 mg/mL in DMSO) were added to the cells at the indicated concentrations.

### III. Measurement of Glucose Uptake Activity, GLUT4 Translocation and AMPK Phosphorylation

Glucose uptake activity in L6 myotubes treated with cacao liquor procyanidins was measured using 2-[<sup>3</sup>H]-deoxy-D-glucose ([<sup>3</sup>H]-2DG, American Radiolabeled Chemicals Inc., St Louis, MO, USA) as described previously<sup>(12)</sup>. To estimate GLUT4 translocation, myotubes were treated with each procyanidin fraction (0.01 - 10 µg/mL), insulin (100 nM) or DMSO as a vehicle control for 15 min. Cell lysate and the cytoplasm and plasma membrane fractions were prepared as previously

described<sup>(12)</sup>. GLUT4 translocation and AMPK-phosphorylation were detected by western blot analysis. Anti-GLUT1, anti-GLUT4, anti-β-actin and horseradish peroxidase-conjugated anti-goat, anti-rabbit and anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-AMPKα, anti-phospho-AMPKα antibodies were from Cell Signaling Technology (Danvers, MA, USA).

### IV. Measurement of α-Glucosidase Activity in the Small Intestine.

To measure the inhibitory effect of CLPr and low- and high-DP fractions at 0.01 to 0.1 µg/mL (final concentration) on α-glucosidase *in vitro*, the α-glucosidase activity was measured using acetone powder of rat intestine as previously described<sup>(13)</sup>. The percent of inhibition was calculated by the following formula and the IC<sub>50</sub> value was calculated:

$$\% \text{ inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

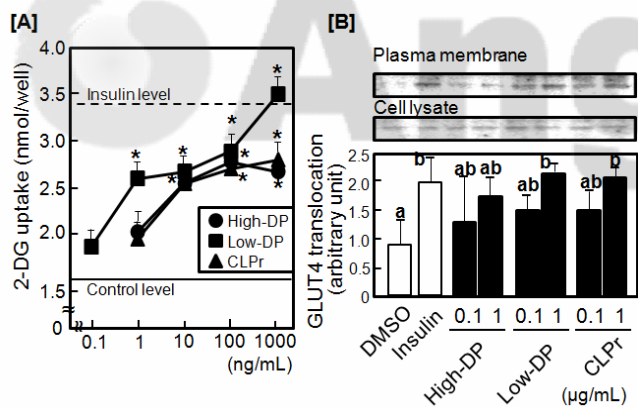
As, is the difference in absorbance decrease at 505nm between a blank and the sample; Ac, is the absorbance of the control.

## RESULTS AND DISCUSSION

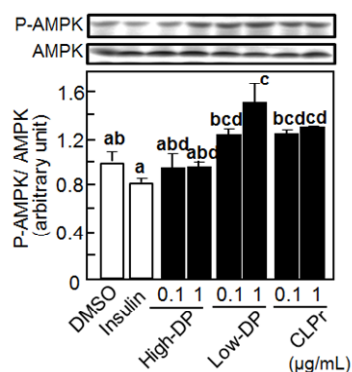
### I. Cacao Liquor Procyanidins Stimulate Glucose Uptake Accompanied by GLUT4 Translocation in Muscle Cells

We first investigated the effects of CLPr and low- and high-DP fractions on glucose uptake and GLUT4 translocation in L6 myotubes. Insulin increased glucose uptake by approximately 1.8-fold of the DMSO-treated negative control (Figure 1A). In the absence of insulin, all procyanidin fractions increased glucose uptake in a dose-dependent manner. Low-DP fraction showed the strongest effect and significant increase was observed at 1 ng/mL. The maximum effect was observed at 1 µg/mL and the extent of increase at this concentration was a similar response to 100 nmol/L insulin. In the case of CLPr and high-DP fraction, significant increase was observed at 10 ng/mL and 100 ng/mL, respectively.

Under the same experimental conditions, we confirmed that insulin significantly promoted GLUT4 translocation in L6 myotubes (Figure 1B). Regarding the cacao liquor procyanidins, low-DP fraction and CLPr at 1 µg/mL significantly promoted GLUT4 translocation. These results indicated that low-DP fraction was more effective than high-DP one. In contrast, the amount of GLUT1 in the plasma membrane was unchanged (data not shown). Moreover, all fractions did not affect the expression level of GLUT4 in the cell lysate (Figure 1B).



**Figure 1.** Effects of cacao liquor procyanidins on promotion of glucose uptake and GLUT4 translocation in L6 myotubes. L6 myotubes were treated with CLPr and low-DP and high-DP fractions at 0.1n-1 $\mu$ g/mL, 100 nM insulin or DMSO for 15min. [A], 2DG uptake activity was measured, and data are expressed means  $\pm$  SE (n = 3). Dotted and solid lines represent control and insulin levels, respectively. Asterisks indicate significant different from the DMSO by Dunnett's test ( $p < 0.05$ ). [B], typical results of GLUT4 protein by western blot (the upper and middle panels). The bottom panel showed the density of each band was analyzed (n = 3) and normalized to that of GLUT1 (data not shown). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ( $p < 0.05$ ).



**Figure 2.** Effects of cacao liquor procyanidins on AMPK phosphorylation in L6 myotubes. L6 myotubes were treated with cacao liquor procyanidins at 0.1 and 1  $\mu$ g/mL, 100 nM insulin or DMSO for 15 min. The upper panels showed typical result of p-AMPK and AMPK by western blot. The bottom panel showed that phosphorylation ratio of AMPK after analysis of the density of each band (n = 3). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ( $p < 0.05$ ).

It is known that the AMPK-dependent pathway is involved in GLUT4 translocation. Thus, we examined the effects of cacao liquor procyanidins on the phosphorylation of AMPK. As a result, low-DP fraction and CLPr at 1  $\mu$ g/mL stimulated AMPK phosphorylation significantly. On the other hand, high-DP fraction and insulin did not affect AMPK phosphorylation (Figure 2). The expression level of AMPK did not alter in all treatment.

In this study, only 1  $\mu$ g/mL of low-DP fraction, which

was consisting of monomeric, dimeric and trimeric procyanidins, showed stronger effects on glucose uptake activity comparable to 100 nM insulin (Figure 1). Our previous report demonstrated that epicatechin at 5  $\mu$ M stimulated glucose uptake, but its activity was weaker than 100 nM insulin<sup>(12)</sup>. Therefore, the activities of dimeric and trimeric compounds are stronger than epicatechin. From these results, dimeric and trimeric procyanidins mainly contribute to the CLPr-promoted glucose uptake activity accompanied by GLUT4 translocation in L6 myotubes. Previous reports demonstrated that dimeric and trimeric procyanidins have greater bioavailability<sup>(5,14)</sup> and antioxidant activities compared to monomer and polymers<sup>(15,16)</sup>. Therefore, dimeric and trimeric procyanidins are active compounds in procyanidins-rich plant extracts and they are involved in various health promotion effects.

Findings in this study indicate that AMPK-dependent pathway is, at least in part, involved in the GLUT4 translocation (Figure 2). It is known that exercise and contraction of muscle promotes insulin-independent translocation of GLUT4 by activating AMPK. Recent reports demonstrate that certain polyphenols have a potency to activate AMPK. For example, (-)-epigallocatechin gallate (EGCG) stimulated phosphorylation of AMPK in L6 myotubes<sup>(17)</sup>. Procyanidin-rich grape seed extract improved insulin resistance through the AMPK phosphorylation in muscle of rats<sup>(18)</sup>. However, this report did not estimate GLUT4 translocation in muscle. Therefore, our findings in current study is meaningful for prevention hyperglycemia by procyanidins, because we clarified the procyanidin-stimulated AMPK activation participated in promoting glucose uptake and GLUT4 translocation in skeletal muscle.

## II. Cacao Liquor Procyanidins Inhibit $\alpha$ -Glucosidase Activity in the Small Intestine

Next, we measured the inhibitory effects of cacao liquor procyanidins on  $\alpha$ -glucosidase activity *in vitro* (Table 1). All fractions inhibited maltase and sucrase-isomaltase in a dose-dependent manner with the IC<sub>50</sub> values around 50  $\mu$ g/mL. High-DP fraction showed the strongest inhibitory effect on the maltase activity which mainly contributes to rising postprandial blood glucose level. Our results are consistent with the previous results: Higher degree of polymerized procyanidins from grape seed and pine bark inhibited intestinal  $\alpha$ -glucosidase activity *in vitro*<sup>(19,20)</sup>. The inhibitory effect was dependent on the degree of polymerization, and tetrameric and more polymerized procyanidins revealed the strongest inhibition of  $\alpha$ -glucosidase activity<sup>(19)</sup>.

Taken together, these results indicate that cacao liquor procyanidins have anti-hyperglycemic activity with different mechanisms depending on the degree of polymerization: Low-DP procyanidins promote glucose uptake activity in muscle whereas high-DP procyanidins inhibit maltase in small intestine. Further study is needed to clarify underlying molecular mechanisms in detail.

**Table 1.** The IC<sub>50</sub> of cacao liquor procyanidins on  $\alpha$ -glucosidases

$\alpha$ -Glucosidase activity IC <sub>50</sub> ( $\mu$ g/mL)	CLPr		
	High-DP (DP > 3)	Low-DP (DP $\leq$ 3)	Whole
Maltase	43.7 $\pm$ 1.8	56.2 $\pm$ 1.4	50.3 $\pm$ 4.0
Sucrase-isomaltase	59.8 $\pm$ 2.8	50.4 $\pm$ 0.8	59.2 $\pm$ 0.5

Values are means and SE (n = 3).

## CONCLUSIONS

Cacao liquor procyanidins possess anti-hyperglycemic activities with different mechanisms depending on the degree of polymerization. Low-DP procyanidins may promote glucose uptake activity by inducing GLUT4 translocation through the AMPK-dependent pathway in muscle, while high-DP procyanidins may delay glucose absorption by inhibiting  $\alpha$ -glucosidase in small intestine.

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