DOI:10.1021/jf100538t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Beneficial Effects of Cinnamon Proanthocyanidins on the Formation of Specific Advanced Glycation Endproducts and Methylglyoxal-Induced Impairment on Glucose Consumption

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Advanced glycation endproducts (AGEs) are a group of complex and heterogeneous compounds formed from nonenzymatic reactions. The accumulation of AGEs in vivo has been implicated as a major pathogenic process in diabetic complications and other health disorders, such as atherosclerosis and Alzheimer's disease, and normal aging. In this study, we investigate the inhibitory effects of cinnamon bark proanthocyanidins, catechin, epicatechin, and procyanidin B2 on the formation of specific AGE representatives including pentosidine, N^{ε}-(carboxymethyl)lysine (CML), and methylglyoxal (MGO) derived AGEs. These compounds displayed obvious inhibitory effects on these specific AGEs, which are largely attributed to both their antioxidant activities and carbonyl scavenging capacities. Meanwhile, in terms of their potent MGO scavenging capacities, effects of these proanthocyanidins on insulin signaling pathways interfered by MGO were evaluated in 3T3-L1 adipocytes. According to the results, proanthocyanidins exerted protective effects on glucose consumption impaired by MGO in 3T3-L1 fat cells.

KEYWORDS: Proanthocyanidin; cinnamon bark; pentosidine; N^{ε}-(carboxymethyl)lysine (CML); MGO; insulin signaling

INTRODUCTION

Advanced glycation endproducts (AGEs) are a class of complicated sugar-derived substances formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, nucleic acids, and others. Recently, the accumulation of AGEs *in vivo* has been implicated as a major pathogenic process in diabetic complications and other health disorders, such as atherosclerosis and Alzheimer's disease, and normal aging (*1*, *2*). Though the chemical nature of AGEs is largely unknown, there is a growing number of structurally defined AGE adducts that have been detected in human tissues (*3*). The best chemically characterized AGEs in humans are pentosidine and N^{ε}-(carboxymethyl)lysine (CML) (*4*). Pentosidine is a fluorescent cross-link with visible wavelength fluorescence derived from the reaction of lysine and arginine with several carbohydrate precursors including glucose, ribose, and pentose (*5*, *6*). Pentosidine has been found to increase gradually in tissue collagens and other tissue proteins with age (*7*), and its levels in the skin and serum have been observed at accelerated rates in diabetics (*8*). Different from pentosidine, CML is a nonfluorescent and noncross-linking derivative related to lysine. As a glycoxidation product, CML accumulates in tissues with age (*9*) and has been detected at elevated levels in patients with diabetes (*10*), making it an integrative biomarker for general oxidative stress, atherosclerosis, and diabetes in humans (*11*, *12*). Besides, methylglyoxal (MGO)-derived AGEs also serve as examples of well-characterized AGEs. MGO is a highly reactive α-oxoaldehyde formed endogenously in numerous enzymatic and nonenzymatic reactions (*13*). The reactions between MGO and various amino residues in proteins not only result in inactivation of enzymes but also lead to the formation of different detrimental AGEs (*14*). For example, it has been found that the cysteine residue of creatine kinase (CK, EC 2.7.3.2) could be subjected to MGO-induced glycation, leading to the reduction or even loss of its activity when incubated with MGO under physiological conditions (*15*). During the process of CK glycation caused by MGO, CK cross-links have been detected and identified as monomers, dimers, and trimers (*15*).

In our previous work, several proanthocyanidins including catechin, epicatechin, and procyanidin B2 from cinnamon bark were identified as AGE inhibitors, which significantly inhibited the formation of total fluorescent AGEs in the BSA-glucose model (*16*). In this study, we continued to evaluate their effects on specific and typical AGEs as mentioned above (pentosidine, CML, and MGO-induced CK cross-links) in order to further understand their inhibitory actions. In addition, MGO was reported to

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 Published on Web 05/17/2010

be one of the important factors to cause insulin resistance. Elevated levels of MGO have been observed in different insulin resistance states, such as in diabetes patients (*17*) and hypertensive animals (*18*). Increased accumulation of endogenous MGO could further impair insulin-stimulated signaling, disturb glucose metabolism, and decrease insulin-induced glucose uptake, which may promote the pathophysiology of diabetes in general (*19*). It has been reported that certain MGO scavengers including aminoguanidine (AG) and *N*-acetyl cysteine could hamper MGO-associated impairment of insulin signaling pathways (*19*). Therefore, it is also of great interest to investigate whether proanthocyanidins with their potent MGO scavenging capacities (*16*) possess possible protective effects on insulin signaling pathways from the influence of MGO in cell culture.

MATERIALS AND METHODS

Chemicals and Instrumentation. Aminoguanidine (AG), sodium azide, bovine serum albumin (BSA), D-glucose, D-ribose, phosphate buffered saline (PBS, pH 7.4), 2,2′-azino-bis (3-ethylbenzthiazoline)6-sulfonic acid (ABTS), potassium peroxodisulfate, trolox, adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine kinase (from rabbit muscle, CK), methylglyoxal (MGO) (40% aqueous solution), horseradish peroxidase (HP), glucose oxidase (GOD), 4-aminoantipyrine (AA), *N*-ethyl-*N*-sulfopropyl-*m*-toluidine (EST), catechin, epicatechin, dexamethasone, and indomethacin were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal to carboxymethyllysine (CML) antibody and goat polyclonal to rabbit IgG were purchased from Abcam Company (Cambridge, UK). Creatine and 5 mM borate buffer ($Na_2B_4O_7 \cdot 10H_2O$, pH 9) were purchased from Hengxin Chemical Reagent Company (Shanghai, China). Tetrabutylammonium bromide, magnesium acetate, and triethylamine were obtained from Kermel Chemical Reagent Company (Tianjin, China). Procyanidin B2 (reference standard) was purchased from Extrasynthese (Genay, France). 3T3-L1 fibroblasts and Dulbecco's modified Eagle's medium (DMEM) were obtained from American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Brooklyn, Australia). Antibiotic−antimycotic and insulin (human recombinant) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). All analytical and HPLC grade solvents used were obtained from BDH Laboratory Supplies (Poole, UK). Absorbance in the enzyme-linked immunosorbent assay (ELISA) was determined by a microplate reader (TECAN Sunrise, Crailsheim, Germany). Fluorescent intensity was measured by a Hitachi F-2500 fluorescent spectrometer (Hitachi Corporation, Tokyo, Japan). Analytical HPLC was carried out using a Shimadzu LC-20AT system equipped with a diode array detector and LC-Solution software. Glucose concentration in cell medium was determined by a colorimetric method on a Shimadzu UV-1206 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Antioxidant Activity Measurement. Total antioxidant capacity was determined using the trolox equivalent antioxidant capacity (TEAC) assay according to the literature (*20*). Briefly, 7 mM ABTS salt solution was reacted with 2.45 mM potassium peroxodisulphate solution, and the reaction mixture was allowed to stand in the dark for 16 h at room temperature and was used in two days. The resultant radical solution was diluted with deionized water to an absorbance of 0.7 ± 0.05 at 734 nm. Fifty microliters of diluted sample solution (in PBS, except epicatechin, which was dissolved in a small amount of DMSO first and then diluted in PBS) or standard (different concentrations of trolox) was added to 1.9 mL of diluted $ABTS^{\cdot+}$ solution, and absorbance was taken at 734 nm on a UV-1206 spectrophotometer (Shimadzu, Kyoto, Japan) after 6 min of incubation. Results were expressed as TEAC values (μmol trolox/μmol compound). Triplicate analyses were carried out.

Evaluation of the Inhibitory Effect of Proanthocyanidins on Pentosidine Formation. The presence of pentosidine was characterized by a typical fluorescence with excitation and emission maxima at 335 and 385 nm, respectively (*21*). Briefly, 5 g of BSA and 14.4 g of D-glucose were dissolved in phosphate buffered saline (pH 7.4) to obtain the control solution with 50 mg/mL BSA and 0.8 M D-glucose. The control solution also contained 0.2 g/L NaN_3 to ensure an aseptic condition. The control solution (2.76 mL) was incubated at 37 °C for 7 days in the presence or absence of 0.24 mL of testing compounds (final concentrations are 1 mM for AG and 100 μM for different proanthocyanidins). After 7 days of incubation, fluorescent intensity was measured for different test solutions. Percent inhibition of pentosidine formation by each compound was calculated using the following equation: % inhibition = [1 − (fluorescence of the solution with inhibitors/fluorescence of the solution without inhibitors)] × 100%.

Evaluation of the Inhibitory Effect of Proanthocyanidins on CML Formation. The measurement of CML content was conducted using the enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to the method described by Mera et al. (*22*) with slight modifications. Briefly, 2 mg/mL BSA was incubated with 33 mM ribose in phosphate buffered saline (pH 7.4) at 37 °C for 7 days in the absence and presence of different compounds (1 mM for AG and 100 μM for different proanthocyanidins). NaN_3 (0.2 g/L) was added into the test solution to ensure aseptic conditions. After incubation, antigen was diluted to 10 μg/mL in 50 mM sodium carbonate buffer (pH 9.5−9.7) and loaded in a 96-well polystyrene plate (0.2 mL per well). The plate was coated overnight at 4 °C. After coating, wells were washed three times with PBS and then blocked with of 0.5% (w/v) gelatin solution (0.3 mL per well) for 3 h at room temperature. Rabbit polyclonal antibody to CML was diluted at a titer of 1:500 in PBS containing 1% (w/v) BSA and loaded in each well (0.1 mL per well). After the overnight incubation at 4 °C, wells were washed three times with 0.05% (v/v) NP-40 solution. An alkaline phosphatase-conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of 1:1000 in PBS containing 1% (w/v) BSA, incubated for 3 h at room temperature and washed with 0.05% (v/v) NP-40 solution again. The wells were subsequently added with *p*-nitrophenyl phosphate substrate solution (pH 10.4, 0.1 mL per well), allowing color to develop at room temperature for 15 min. Then, the absorbance at 405 nm was determined with a microplate reader. Percent inhibition of CML formation by each compound was calculated using the following equation, % inhibition = [1 − (absorbance of the solution with inhibitors/absorbance of the solution without inhibitors)] × 100%.

Evaluation of the Inhibitory Effect on Cysteine-Derived AGEs. Owing to the difficulties in quantifying MGO-induced CK cross-links, the determination of CK activity can be used as an alternative way to reflect amounts of CK cross-links as the formation of MGO-caused cross-links correlates with the decrease in CK activity, which behaved in a dose-dependent manner (*15*). Here, we employed ion-pair high-performance liquid chromatography to evaluate CK activity according to the generation rate of ADP derived from ATP in CK reaction exactly as we published before (*23*). All sample solutions were prepared in 5 mM borate buffer (pH 7.4). Borate buffer (0.8 mL) (pH 7.4) was added with mixture of 10 μL of 5 mM compounds (AG, catechin, epicatechin, and procyanidin B2) or control (10 μL borate buffer, pH 7.4), 10 μL of 11.5 μM CK, and 10 μL of 5 mM MGO, and then were mixed together and incubated under 37 °C for 2 h. Subsequently, samples were added with 100 μL of reaction solution (24 mM creatine, 4 mM ATP, and 5 mM magnesium acetate). Besides, the sample solution containing only CK was prepared as the control. Using the same HPLC method as we published before (*30*), CK activities in different samples were measured.

Cell Culture. 3T3-L1 fibroblast cells were maintained in DMEM containing 25 mM glucose, supplementing with 10% fetal bovine serum, and 1% antibiotics. Two days after seeding into 12-well plates, the cells reached about 80% confluence. Cells were induced to differentiate to adipocytes by switching the medium to differentiation medium (DMEM) supplemented with 10% fetal bovine serum, 5 μg/mL human recombinant insulin, 1 μM dexamethasone, 125 μM indomethacin, and 1% antibiotics for 3 days. Then, cells were grown in various transition media for 7 days, which were composed of DMEM containing 10% fetal bovine serum, 5 μg/mL human recombinant insulin, 1% antibiotics, and different concentrations of MGO together with or without different proanthocyanidins, respectively. The concentrations for MGO and proanthocyanidins in transition medium were MGO (39 μM, 78 μM, and 156 μM), proanthocyanidins (36 μM), and MGO (78 μM) plus proanthocyanidins (36 μM). The transition medium was changed every two days. Twenty microliters of each transition medium was collected at different time points (at 24 h on 3, 5, and 7 days of transition period) and was subsequently diluted with phosphate buffer to reach a total volume of 400 μL. Glucose concentration in the medium was determined according to the approach mentioned below.

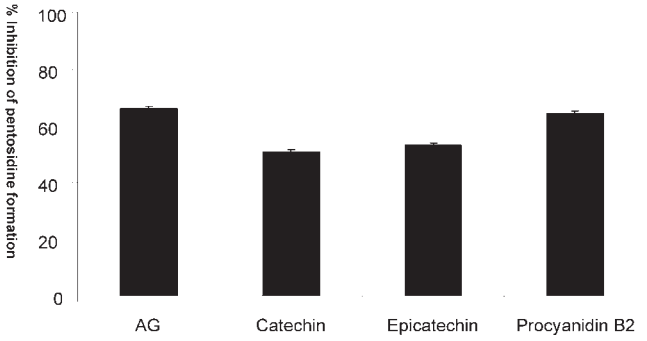

Figure 1. Inhibitory effects of various compounds on the formation of pentosidine. Concentrations of all proanthocyanidins are 100 μM and that of AG (positive control) is 1 mM. Results are means $\pm$ SD for $n = 3$ and $P < 0.05$.

Determination of Glucose Consumption Ability. The consumption ability of the medium glucose of 3T3-L1 adipose cells was determined as a reference for the glucose metabolism situation which is regulated by complex insulin signaling pathways. Glucose concentration in the cell medium was determined on the basis of the method described in the literature (*24*) with little modification. Briefly, 10 mM stock solutions of 4-aminoantipyrine (AA) and *N*-ethyl-*N*-sulfopropyl-*m*-toluidine (EST) were freshly prepared in water. Subsequently, AA and EST stocks were mixed together (1:1, v/v) and diluted with 0.8 M sodium phosphate buffer, pH 6.0, to obtain a chromophore stock in which the final concentrations of AA and EST were both 3.5 mM. Horseradish peroxidase (HP) and glucose oxidase (GOD) stocks used in our study were freshly prepared in water at concentrations of 0.48 mg/mL (54 units/mL) and 5.5 mg/mL (31 units/mL), respectively. Assays were routinely conducted by mixing 500 μL of the chromophore stock, 50 μL of HP stock, 50 μL of diluted cell medium (20-fold dilution), 350 μL of water, and 50 μL of GOD stock to make a total volume of 1 mL. After incubation for 1 h at 25 °C, we determined the absorbance of each sample at 550 nm. Quantification was based on a standard curve generated from different concentrations of glucose solutions. Glucose consumption ability in this study was defined as glucose consumption amount/time (mM/24 h), where the glucose consumption amount refers to the decrease of glucose amount in media when compared to the glucose level in the initial transition medium.

Statistical Analysis. Statistical analyses were performed using the SPSS statistical package (SPSS, Inc., Chicago, IL, USA). Paired sample *t* test was applied to determine whether a particular treatment of the sample would cause a significant difference when compared to the control. $P < 0.05$ was selected as the level decision for significant differences.

RESULTS AND DISCUSSION

Inhibitory Effects on Pentosidine Formation. Pentosidine has been recognized as a fluorescent protein cross-link from human extracellular matrix, which involves lysine and arginine residues. The system composed of glucose and BSA was employed as a model leading to the formation of pentosidine in our study. Virtually, pentosidine can also be formed from other sources, such as fructose, ascorbate, Amadori compounds, and 3-deoxyglucosone, which may provide relatively lower yields (*25*). As shown in **Figure 1**, catechin, epicatechin, and procyanidin B2 exhibited strong inhibitory effects (more than 50% inhibition percentage) on the formation of pentosidine. Procyanidin B2 possessed the highest inhibitory ability (nearly 70% inhibition percentage) and was comparable to that of AG, a positive control, which was used at the concentration of 1 mM. Referring to their antioxidant activities (data not shown), it seems the inhibitory effects of proanthocyanidins on the formation pentosidine are highly correlated with their antioxidative capacities, suggesting that the inhibitory action of proanthocyanidins on pentosidine formation may mainly be contributed by their strong antioxidative activities.

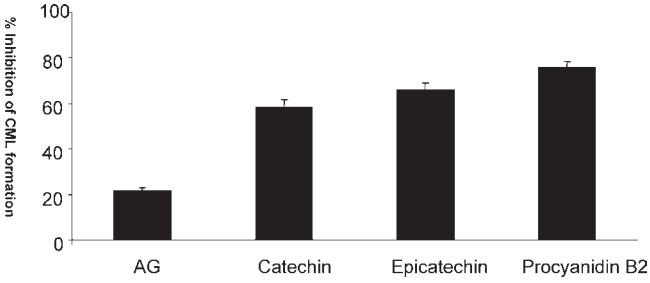

Figure 2. Inhibitory effects of various compounds on the formation of CML. Concentrations of all proanthocyanidins are 100 μM and that of AG (positive control) is 1 mM. Results are means $\pm$ SD for $n = 3$ and $P < 0.05$.

Inhibitory Effects on CML Formation. As shown in **Figure 2**, all proanthocyanidins displayed obvious inhibitory effects (more than 50% inhibition percentage) on the formation of CML in the system composed of BSA and ribose. Particularly, procyanidin B2, a dimer-type proanthocyanidin, showed the highest inhibition capacity (almost 80% inhibition). CML has been confirmed to be a derivative of lysine with several pathways including the oxidative cleavage of fructoselysine formed from sugar and lysine, rearrangement of dicarbonyl-modified lysine (*26*), and reaction between lysine and products of autoxidation of ascorbate (*27*). In our study, CML was formed in the model system composed of BSA and ribose, thus basically through the former two pathways mentioned above. As the oxidative process is mainly involved in the formation of CML, antioxidant activity of proanthocyanidins may play an important role in the inhibition of CML formation, which has been confirmed by the correlation between their inhibitory effects on CML formation and their antioxidant activities (data not shown). Meanwhile, CML can be also formed from the reactions between dicarbonyls (such as glyoxal and MGO) and lysine, and compounds with scavenging capacities on these dicarbonyls are supposed to affect the formation of CML. Thus, as a potent MGO-scavenger, AG with weak antioxidant activity showed obvious inhibitory effects on CML formation, while proanthocyanidins are supposed to inhibit CML formation through both their strong antioxidant activities and dicarbonyl scavenging abilities (*16*).

Inhibitory Effects on MGO-Induced Cross-Links in CK. It was reported that MGO could lead to the inactivation of CK via glycation to its active cysteine residues (*15*), and the resultant MGO−cysteine cross-link is also viewed as a kind of AGE. With the increase of MGO, the level of MGO−cysteine cross-link enhanced, while CK activity decreased (**Figure 3a**), suggesting that the amount of MGO−cysteine cross-links can be regarded as inversely correlated with CK activity. Hence, the examination of their protective effects on CK activity impaired from MGO can be used as an approach to investigate the inhibitory effects of compounds on the MGO−cysteine cross-link. For this consideration, all compounds were first incubated with CK to observe their effects on CK activity. Proanthocyanidins exhibited more or less suppressive effects on CK activity, while AG showed a neglectable effect (**Figure 3b**). It has been reported that certain polyphenols, such as tannins, are able to interact with proteins through covalent or noncovalent binding, thus affecting proteins in structure and function (*28*). Therefore, inhibitory effects of proanthocyanidins on CK activity may be due to the protein−phenol association. However, these proanthocyanidins still demonstrated obvious protective effects on CK activity under incubation with MGO (**Figure 3c**), which suggests that they could abate the influence of MGO on CK and consequently reduce the formation of MGO cross-links.

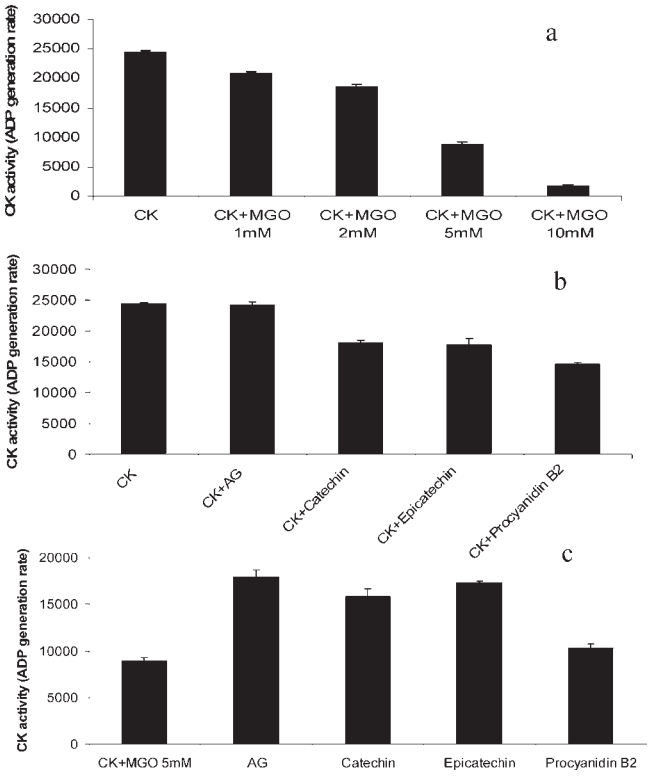

Figure 3. Effects of MGO and proanthocyanidins on CK activity. (**a**) Influences of different concentrations MGO on CK activity. (**b**) Effects of proanthocyanidins on CK activity. The concentration of CK is 11.5 μM, while concentrations of proanthocyanidins and AG are 5 mM. (**c**) Effects of proanthocyanidins on CK activity impaired by MGO. The concentration of CK is 11.5 μM and concentrations of proanthocyanidins, AG, and MGO are 5 mM. Results are means $\pm$ SD for $n = 3$ and $P < 0.05$.

Protective Effects of Proanthocyanidins on Glucose Consumption Impaired by MGO in 3T3-L1 Adipocytes. Insulin-mediated signaling pathways play an important role in a series of cellular activities including controlling cell metabolism, growth, and differentiation. Impairment of this hormone's metabolic events results in insulin resistance, which is related to various diseases such as diabetes, obesity, and atherosclerosis. Studies have suggested that an increase in intracellular MGO inhibits insulin signaling and leads to insulin resistance (*29*). As insulin-stimulated signaling pathways are of vital importance in regulating glucose metabolism, the glucose consumption ability of fat cells could be regarded as a representative index to describe the insulin signaling status. Thus, when insulin signaling pathways are impaired, the glucose consumption ability of fat cells would be weakened accordingly.

Results indicated that no obvious difference of glucose consumption ability was observed among cells grown in treated transition media on the third and fifth day of the transition period (data not shown). However, on the seventh day of the transition period (**Figure 4**), it was apparent that glucose consumption ability was reduced consequently with the elevated concentration of MGO added into the transition medium. At the same time, proanthocyanidins could reserve the glucose consumption capacities of fat cells grown in media added with MGO, suggesting that they could protect insulin signaling pathways to some extent though proanthocyanidins themselves seemed to weaken the glucose consumption ability of cells more or less, except for catechin. As mentioned before, some polyphenols could bind to proteins in reversible or irreversible ways to form covalent or noncovalent linkages, leading to precipitation and aggregation of proteins (*30*).

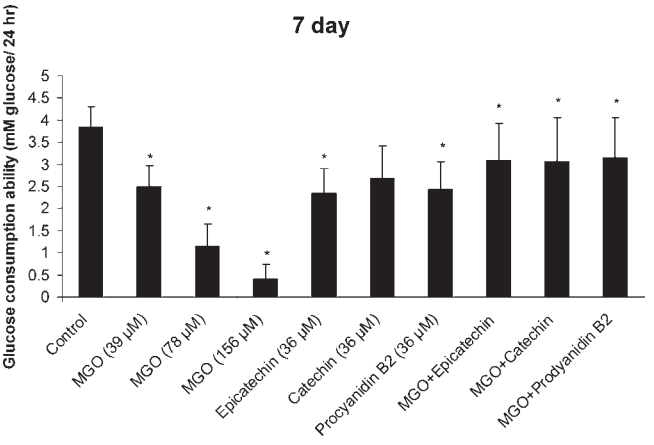

Figure 4. Glucose consumption ability of cells grown in transition media (7 day transition period) added with different concentrations of MGO together with or without proanthocyanidins. Seventy-eight micromolar MGO was added together with 36 μM proanthodyanidins. Results are expressed as means $\pm$ SD for $n = 3$. Bars with an asterisk indicate a significant difference from the control ($P < 0.05$).

Hence, effects of proanthocyanidins on glucose consumption abilities may result from their potential interaction with proteins involved in insulin signaling pathways.

The harmful effects brought by MGO on glucose consumption may be closely related to its modification to insulin or other involved insulin-associated receptors. It has been observed that structure and function of human insulin would be affected after incubation with MGO in vitro. MGO mainly modifies the B-chain of human insulin, causing the decrease of insulin activity in the aspect of insulin-mediated glucose uptake by target cells or tissues (*31*). Thus, abnormalities of the insulin molecule in structure and function induced by MGO may cause the pathogenesis of insulin resistance (*32*). Besides, MGO was reported to easily react with intracellular proteins, nucleotides, and basic phospholipids, leading to damages to the proteome, genome, and lipidome (*33*). In this notion, MGO could affect cellular responses, implying its role in cell signaling, aging, and relevant diseases. Hence, it is highly likely that MGO would interfere with other additional actors in the insulin signaling pathways. A recent study has found that MGO could make a conformational change to IRS proteins which are important insulin receptor substrates in insulin signaling with a slow turn rate (*34*) through direct binding and thus affecting their docking functions. However, effects of MGO on other constituents of insulin signaling need further investigation.

In conclusion, our findings demonstrate that proanthocyanidins such as catechin, epicatechin, and procyanidin B2 identified from cinnamon bark possessed obvious inhibitory effects on the formation of three typical AGEs (pentosidine, CML, and MGO-induced cross-link in CK), which were mainly contributed by both their strong antioxidant activities and carbonyl scavenging abilities. However, as potential MGO scavengers with benefits to human health, proanthocyanidins examined in the work exerted distinct protective effects on glucose consumption impaired by a higher concentration of MGO. It is worth noting that proanthocyanidins themselves may slightly affect glucose consumption due to their possible interaction with certain proteins engaging in insulin signaling. Since detailed mechanisms of the reactions between proanthocyanidins and proteins are not clear, it is necessary to have a further evaluation of positive actions of proanthocyanidins on MGO-induced detrimental impacts on insulin signaling.

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Received for review February 12, 2010. Revised manuscript received April 7, 2010. Accepted May 10, 2010.