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Effects of *C*-glycosylation on anti-diabetic, anti-Alzheimer's disease and anti-inflammatory potential of apigenin

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ABSTRACT

Apigenin has gained particular interests in recent years as a beneficial and health promoting agent because of its low intrinsic toxicity. Vitexin and isovitexin, naturally occurring *C*-glycosylated derivatives of apigenin, have been known to possess potent anti-diabetic, anti-Alzheimer's disease (anti-AD), and anti-inflammatory activities. The present study was designed to investigate the anti-diabetic, anti-AD, and anti-inflammatory potential of apigenin and its two *C*-glycosylated derivatives, vitexin and isovitexin by *in vitro* assays including rat lens aldose reductase (RLAR), human recombinant aldose reductase (HRAR), advanced glycation endproducts (AGEs), protein tyrosine phosphatase 1B (PTP1B), acetylcholinesterase (AChE), butyrylcholinesterase (BChE), β-site amyloid precursor (APP) cleaving enzyme 1 (BACE1), and nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Among them, isovitexin was found as the most potent inhibitor against RLAR, HRAR, AGE, AChE, and BChE while vitexin showed the most potent PTP1B inhibitory activity. Despite the relatively weak anti-diabetic and anti-AD potentials, apigenin showed powerful antiinflammatory activity by inhibiting NO production and iNOS and COX-2 expression while vitexin and isovitexin were inactive. Therefore, it could be speculated that *C*-glycosylation of apigenin at different positions might be closely linked to relative intensity of anti-diabetic, anti-AD, and anti-inflammatory potentials.

1. Introduction

Naturally-occurring flavonoids usually exist as *O*- or *C*-glycosides of flavonoid moiety. The *O*-glycosides possess sugar substituents bound to a hydroxyl of aglycone, usually at 3 or 7 positions, whereas *C*-glycosides possess sugar groups bound to carbon of aglycone usually at C-6 or C-8 by forming a C–C bond, giving them more resistance to acid hydrolysis (Rijke et al., 2006; Yegang et al., 2010). The flavone *C*-glycosides, important members of the flavonoid family present in foodstuffs and nutraceuticals, have been reported to possess a wide range of biological activities. Apigenin (4′,5,7-trihydroxyflavone) is a bioflavonoid, which is found in a wide variety of plants, herbs, vegetables and fruits (Havsteen, 2002). On the other hand, flavones *C*-glycosides such as vitexin and isovitexin occur frequently in many edible or medicinal plants including Mung beans (*Vigna radiata*), pigeon pea leaves (*Cajanus cajan* Millsp.), bamboo leaves (*Phyllostachys nigra* var. henonis), *Ficus deltoidea* leaves as the main constituents. They have a wide range of biological activities, including antioxidant, anti-inflammatory, anti-diabetic, neuroprotective, hepatoprotective, and antimicrobial (Kim et al., 1998; Esmail et al., 2012; Choo et al., 2012; Fu et al., 2008; Agnese et al., 2001; Zhang et al., 2005; Zheng et al., 2004).

Although the exact cause of Alzheimer's disease (AD) remains elusive, mounting evidences continues to support the involvements of inflammation, oxidative/nitrosative stress, lack of cholinergic transmission, and presence of β-amyloid plaques as well as

Abbreviations: ACh, acetylthiocholine iodide; AChE, acetylcholinesterase; AGEs, advanced glycation endproducts; AR, aldose reductase; ARI, aldose reductase inhibitor; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride; AD, Alzheimer's disease; Aβ, amyloid-β peptides; NADPH, β-nicotinamide adenine dinucleotide phosphate; APP, β-site amyloid precursor; BACE1, β-site amyloid precursor cleaving enzyme 1; BCh, butyrylthiocholine chloride; BChE, butyrylcholinesterase; ChAT, choline acetyltransferase; COX-2, cyclooxygenase-2; DMEM, Dulbecco's Modified Eagle's Medium; DTNB, 5,5′-dithiobis [2-nitrobenzoic acid]; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HRP, horseradish peroxidase; HRAR, human recombinant aldose reductase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; RLAR, rat lens aldose reductase; ROS, reactive oxygen species; pNPP, *p*-nitrophenyl phosphate; PVDF, polyvinylidene fluoride; PTP1B, protein tyrosine phosphatase 1B.

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neurofibrillary tangles in the pathogenesis of AD (Torreilles et al., 1999; Tiraboschi et al., 2002; Butterfield et al., 2007). Besides these crucial factors involved in the pathogenesis of AD, several studies have also suggested a close link between diabetes mellitus (DM) and AD. Since insulin plays a critical role in maintaining metabolic, neurotrophic, neuromodulatory, and neuroendocrine responses, as well as in memory and learning processes, the impaired insulin signaling in diabetic patients is now considered with underlying DM-associated cognitive declines and dementia by facilitating Aβ and tau protein accumulation and by exerting detrimental effects on neuronal function and survival (Adamo et al., 1989; de la Monte, 2009; Neumann et al., 2008). Moreover, diabetic encephalopathy associated with neurotoxicity from hyperactivation of polyol and advanced glycation endproduct (AGE)/receptor for AGE (RAGE) pathways, indicate persistent hyperglycemia which represents the most relevant risk factor for cognitive dysfunction and increases dementia and AD consequently (Sima, 2010). Several epidemiological studies have demonstrated that chronic inflammation and uncontrolled immune responses are closely linked with a wide variety of diseases, including DM, AD, arthritis, pulmonary diseases, and autoimmune diseases (Lin and Tang, 2008). On the other hand, the brain inflammation, mediated by microglia and astrocyte activation, which produces more inflammatory mediators as a part of aging process, is also a key feature of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases (Lau et al., 2007).

Therefore, considering the interrelationships between DM, AD, and inflammation, the present study is designed to investigate the effects of *C*-glycosylation on anti-diabetic, anti-AD, and anti-inflammatory potential of apigenin and its *C*-glycosylated derivatives using several *in vitro* assays including RLAR, HRAR, AGE and PTP1B inhibitory assays for anti-diabetic potential, BACE1, AChE, and BChE inhibitory assays for anti-AD potential, and LPS-stimulated NO production, iNOS and COX-2 expressions in RAW 264.7 cells for anti-inflammatory potential.

2. Materials and methods

2.1. Reagents

Electric-eel acetylcholinesterase (AChE, EC3.1.1.7), horse-serum butyrylcholinesterase (BChE, EC 3.1.1.8), acetylthiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), *p*-nitrophenyl phosphate (pNPP), ethylenediaminetetraacetic acid (EDTA), β-nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA), DL-glyceraldehyde dimer, D-(−)-fructose, D-(+)-glucose, aminoguanidine hydrochloride, quercetin, apigenin, vitexin, isovitexin, LPS from *Escherichia coli*, Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), fetal bovine serum (FBS), and antibiotics were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone (Logan, Utah, USA). Human recombinant aldose reductase (HRAR 0.4 unit) was purchased from Wako Chemicals (Osaka, Japan). The BACE1 FRET assay kit (β-secretase) was purchased from the Pan Vera Co. (Madison, WI, USA). Various primary antibodies (iNOS, COX-2, and β-actin) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billerica, MA, USA). Supersignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). Unless otherwise indicated, all chemicals were purchased from Sigma–Aldrich Co. (St Louis, MO). The structures of apigenin, vitexin, and isovitexin are shown in Fig. 1.

2.2. Assay for RLAR inhibitory activity

In the experiment, the Guidelines for Care and Use of Laboratory Animals, as approved by Pukyong National University (Busan, South Korea), were followed. According to the modified method of Hayman and Kinoshita (1965), rat lens homogenate was prepared. Briefly, the lens was removed from the eyes of Sprague–Dawley rats (Samtako BioKorea, Inc.) weighing 250–280 g. The lenses were homogenized in sodium phosphate buffer (pH 6.2), which was prepared from sodium phosphate dibasic ($Na_2HPO_4{\cdot}H_2O$, 0.66 g) and sodium phosphate monobasic ($NaH_2PO_4{\cdot}H_2O$, 1.27 g) in 100 mL of double distilled water. The supernatant was obtained by centrifugation of the homogenate at 10,000 rpm at 4 °C for 20 min and was frozen until use. A crude aldose reductase (AR), with a specific activity of 6.5 U/mg, was used in the evaluation for enzyme inhibition. The partially purified material was separated into 1.0 mL aliquots, and stored at −40 °C. Each 1.0 mL cuvette contained equal units of enzyme, 100 mM sodium phosphate buffer (pH 6.2), and 1.6 mM NADPH, both with and without 50 mM of the substrate, DL-glyceraldehyde, and an inhibitor (final concentration; 100 μM for the test compound, dissolved in 100% DMSO, and diluted to 0.01–15 μM). The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 4 min period on a Ultrospec®2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). Epalrestat and quercetin, well known aldose reductase inhibitors (ARIs), were used as positive controls. The inhibition percentage (%) was calculated as $[1 - (\Delta A \text{ sample/min} - \Delta A \text{ blank/min})/(\Delta A \text{ control/min} - \Delta A \text{ blank/min})] \times 100$, where ΔA sample/min represents the reduction of absorbance for 4 min with the test sample and substrate, respectively, and ΔA control/min represents the same, but with 100% DMSO instead of a sample. The RLAR inhibitory activity of each sample was expressed in terms of the IC_{50} value (μM), as calculated from the log–dose inhibition curve.

2.3. Assay for HRAR inhibitory activity

The HRAR inhibitory activities were examined according to the method of Nishimura et al. (1991). The reaction mixture was prepared as follows: 100 μL of 0.15 mM NADPH, 100 μL of 10 mM DL-glyceraldehyde, as a substrate, 5 μL of the HRAR, and various concentrations of the samples (0.01–15 μM) in a total volume of 1.0 mL of 100 mM sodium phosphate buffer (pH 6.2). The AR activity was determined by measuring the decrease in NADPH absorption at 340 nm over a 1 min period on a Ultrospec®2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, New Jersey, USA). Quercetin, a well known ARI was used as a positive control. The inhibition percentage (%) was calculated similar to the RLAR assay, except that ΔA sample/min represents the reduction of absorbance for 1 min with the test samples and substrate. The HRAR inhibitory activity of each sample was expressed in terms of the IC_{50} value (μM), as calculated from the log–dose inhibition curve.

2.4. Assay for inhibitory activity of AGE formation

The inhibitory activity of AGE formation was examined according to the modified method of Vinson and Howard (1996). To prepare the AGE reaction solution, 10 mg/mL of bovine serum albumin in 50 mM sodium phosphate buffer (pH 7.4), with 0.02% sodium azide to prevent bacterial growth, was added to 0.2 M fructose and 0.2 M glucose. The reaction mixture (950 μL) was then mixed with various concentrations of the samples (50 μL, final concentration; 4–200 μg/mL for the test compound) dissolved in 10% DMSO. After incubating at 37 °C for 7 days, the fluorescence intensity of the reaction products was determined on a spectrofluorometric detector (FL × 800 microplate fluorescence reader, Bio-Tek Instrument, Inc., Winooski, USA), with excitation and emission wavelengths at 350 nm and 450 nm, respectively. The inhibitory activity of each sample on AGE formation was expressed in terms of the IC_{50} value (μg/mL) in triplicate. The nucleophilic hydrazine compound, aminoguanidine hydrochloride, was used as a reference in the AGE assay.

2.5. Assay for the PTP1B inhibitory activity

The PTP1B inhibitory activity of apigenin derivatives were evaluated using pNPP (Cui et al., 2006). To each of 96 wells (final volume of 100 μL), 40 μL of PTP1B enzyme [0.5 units diluted with a PTP1B reaction buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM DTT] were added with or without samples (test concentration ranging from 0.05 to 50 μg/mL) dissolved in 10% DMSO. The plate was preincubated at 37 °C for 10 min, and then 50 μL of 2 mM pNPP in PTP1B reaction buffer was added. Following incubation at 37 °C for 20 min, the reaction was terminated with the addition of 10 M NaOH. The amounts of *p*-nitrophenyl produced after enzymatic dephosphorylation from pNPP was estimated by measuring the absorbance at 405 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The nonenzymatic hydrolysis of 2 mM pNPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme. The percent inhibition (%) was obtained by the following equation: % inhibition = $(A_c - A_s)/A_c \times 100$, where A_c is the absorbance of the control, and A_s is the absorbance of the sample. Ursolic acid was used as a positive control.

2.6. In vitro BACE1 enzyme assay

The assay was carried out according to the supplied manual with selected modifications. Briefly, a mixture of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/mL), 10 μL of the substrate (750 nM Rh-EVNL-DAEFK-Quencher in 50 mM, ammonium bicarbonate) and 10 μL of the tested samples [final concentration; 100 μM for compounds] dissolved in 10% DMSO was incubated for 60 min at 25 °C in the dark. The proteolysis of two fluorophores (Rh-EVNLDAEFK-Quencher) by BACE1 was monitored by formation of the fluorescent donor (Rh-EVNL) that increased in fluorescence wavelengths at 530–545 nm (excitation) and 570–590 nm (emission), respectively. Fluorescence was measured