Antidiabetic effect of green rooibos (*Aspalathus linearis*) extract in cultured cells and type 2 diabetic model KK-A^y mice

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Abstract Previous studies have demonstrated anti-diabetic effects for rooibos (*Aspalathus linearis*) and aspalathin (ASP), one of its main polyphenols. Rooibos, an endemic plant of South Africa, is well-known for its use as herbal tea. Green ('unfermented') rooibos has been shown to contain more ASP than 'fermented' rooibos tea, currently the major product. In the present study, we investigated the antidiabetic effect of green rooibos extract (GRE) through studies on glucose uptake in L6 myotubes and on pancreatic β -cell protective ability from reactive oxygen species (ROS) in RIN-5F cells. Its in vivo effect was also examined using obese diabetic KK-A^y mice. GRE increased glucose uptake under insulin absent condition and induced phosphorylation of 5'-adenosine monophosphate-activated protein kinase (AMPK) in L6 myotubes as previously demonstrated for ASP. In addition to AMPK, GRE also promoted phosphorylation of Akt,

another promoter of glucose transporter 4 (GLUT4) translocation, in L6 myotubes unlike ASP, suggesting an involvement of GRE component(s) other than ASP in Akt phosphorylation. Promotion of GLUT4 translocation to the plasma membrane by GRE in L6 myotubes was demonstrated by Western blotting analysis. GRE suppressed the advanced glycation end products (AGEs)-induced increase in ROS levels in RIN-5F pancreatic β -cells. Subchronic feeding with GRE suppressed the increase in fasting blood glucose levels in type 2 diabetic model KK-A^y mice. These in vitro and in vivo results strongly suggest that GRE has antidiabetic potential through multiple modes of action.

Keywords AMPK · GLUT4 · Hyperglycemia · L6 myotube · RIN-5F cell · Rooibos

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Abbreviations

AGEs	Advanced glycation end products
AMPK	5'-Adenosine monophosphate-activated protein kinase
ASP	Aspalathin
GLUT4	Glucose transporter 4
GRE	Green rooibos extract
HPLC	High performance liquid chromatography
PPAG	Phenyl pyruvic acid-2- <i>O</i> - β -glucoside
QROB	Quercetin-3- <i>O</i> -robinobioside
ROS	Reactive oxygen species
SB1	Solvent-based aspalathin-enriched green rooibos extract
STZ	Streptozotocin

Introduction

The number of diabetes mellitus patients is increasing globally because of population growth, aging, increasing physical inactivity and prevalence of obesity (Wild et al. 2004). Type 2 diabetes mellitus in particular is recognized as a global public health concern as it is associated with metabolic syndrome and a marked increase in the risk of cardiovascular morbidity and mortality (Alderman et al. 1999; Genuth et al. 2003; Verdecchia et al. 2004). Type 2 diabetes is also characterized by chronic hyperglycemia due to abnormal insulin secretion or insulin receptor. Chronic hyperglycemia and subsequent augmentation of reactive oxygen species (ROS) under high glucose levels increase insulin resistance and aggravate β -cell function, exacerbating type 2 diabetes (Kaneto et al. 2010). The skeletal muscles, as major tissue responsible for insulin-mediated glucose uptake in the post-prandial state, play a significant role in maintaining glucose homeostasis (Saltiel and Kahn 2001). In skeletal muscle, insulin promotes glucose uptake through a signaling process that leads to activation of phosphatidylinositol-3 kinase (PI3 K) and Akt, resulting in increased translocation of glucose transporter 4 (GLUT4) to the plasma membrane (Saltiel and Kahn 2001). Accordingly, the skeletal muscle tissue, being insulin resistant, is an attractive therapeutic target for the treatment of type 2 diabetes. 5'-Adenosine monophosphate-activated protein kinase (AMPK) is another GLUT4 translocation promoter which consists of three subunits, α , β and γ (Towler and Hardie 2007).

Temporary increase in intracellular Ca^{2+} concentration promotes AMPK activation, and two distinct upstream kinases, LKB1 and calmodulin-dependent protein kinase (CaMKK), lead to glucose uptake, which is related to promoted GLUT4 genesis and its translocation to the cell membrane (Sakamoto et al. 2005; Ojuka 2004; Huang and Czech 2007). Among the several sources of ROS production, advanced glycation end products (AGEs), the products of non-enzymatic glycation and oxidation of proteins, play a crucial role in the pathogenesis of diabetic complications (Bucala and Vlassara 1995; Hammes et al. 1999). Several researches have clearly shown that ROS argumentation diminishes β -cell functions as low intrinsic gene expression of antioxidant enzymes in pancreatic cells make them vulnerable to ROS (Lenzen et al. 1996; Tiedge et al. 1997; Robertson et al. 2007).

Some phenolic constituents of rooibos (*Aspalathus linearis*), an endemic South African plant and well-known for its use as herbal tea, have been reported to show antioxidant properties. Its major flavonoid constituent, aspalathin (ASP) (Joubert 1996), displays potent radical scavenging ability compared to many of the other rooibos flavonoids (Snijman et al. 2009). The total antioxidant activity of green ('unfermented') rooibos infusions was found to correlate very well with its ASP content (Joubert et al. 2008). We have reported that ASP itself suppresses the increases in fasting blood glucose levels and improves glucose intolerance in type 2 diabetic model db/db (Kawano et al. 2009) and ob/ob (Son et al. 2013) mice. It enhances glucose uptake through GLUT4 translocation to the plasma membrane via AMPK activation in L6 myotubes and scavenges AGEs-induced ROS in RIN-5F cells (Son et al. 2013). Enhanced glucose uptake by ASP and a solvent-based ASP-enriched green rooibos extract (SB1) has also been demonstrated in C2C12 myotubes, while acute oral administration of both ASP and SB1 to streptozotocin (STZ)-induced diabetic rats significantly reduced the plasma glucose concentration. SB1 tested at 25 mg/kg body weight had a glucose lowering effect comparable to that of 1,1-dimethylbiguanide hydrochloride (metformin) (Muller et al. 2012). The same extract was more effective than extract prepared from fermented rooibos (with comparative low ASP content) in increasing glucose uptake, mitochondrial activity and ATP production in palmitate-induced insulin

resistant C2C12 cells (Mazibuko et al. 2013). Both extracts down-regulated protein kinase C θ (PKC θ) and increased activation of Akt and AMPK and levels of GLUT4. Given these promising results on the glucose lowering ability of ASP and ASP-enriched rooibos extract in different test systems, and their antioxidant effects, further investigation of GRE as potential antidiabetic nutraceutical and ASP as major compound is merited.

For the present study, a cold-water based green rooibos extract (GRE) was chosen as this type of extract is currently produced by industry as food ingredient and for the nutraceutical market. We investigated the effect of GRE and ASP on glucose uptake in cultured muscle cells. The antioxidant ability of GRE on AGEs-induced oxidative stress was determined, using cultured pancreatic β -cells. Additionally, we examined the effect of dietary GRE on attenuating increased fasting blood glucose levels employing type 2 diabetic model KK-A y mice.

Materials and methods

Materials

Spray-dried powder of water-soluble green rooibos extract (GRE, lot no. E2CCL) and aspalathin (ASP) were supplied by Rooibos Ltd. (Clanwilliam, South Africa), and Tama Biochemical Co., Ltd. (Tokyo, Japan), respectively. As authentic reference standards for GRE compositional analysis, ASP and enolic phenylpyruvic acid-2-*O*-glucoside (PPAG) were obtained from the compound library of ARC Infrutec-Nietvoorbij (Stellenbosch, South Africa). The other authentic reference standards, except rutin (Sigma-Aldrich Co., St. Louis, MO, USA), were supplied by Extrasynthese (Genay, France). All standards were of >95 % purity. L6 myoblasts derived from rat skeletal muscle cells and RIN-5F cells derived from rat pancreatic β -cells were obtained from the American Type Culture Collection (Manassas, VA, USA; ATCC[®]) numbers: CRL-1458 and CRL-2058, respectively. Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS, USA). Streptomycin and penicillin G were from Nacalai Tesque, Inc. (Kyoto, Japan). Bovine

serum albumin (BSA, fatty acid-free) and Triton X-100 were purchased from Sigma-Aldrich Co. The glucose assay kit (Glucose CII-Test Wako) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Compound C, an AMPK inhibitor, was from Wako Pure Chemical Industries and 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) was from Toronto Research Chemicals (Toronto, ON, Canada). The anti-phospho-Akt (ser473) antibody was from Upstate Biotechnology (Lake Placid, NY, USA). The anti-Akt1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-AMPK α (Thr172) and anti-AMPK antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-Na $^+$ K $^+$ -ATPase α -1 antibody was from Millipore (Billerica, MA, USA), and anti-GLUT4 antibody was from Sigma-Aldrich Co. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). All other chemicals were of the best grade commercially available, unless otherwise noted. Plastic multiwell plates and tubes were obtained from Nunc A/S (Roskilde, Denmark), or Iwaki brand, Asahi Glass Co., Ltd. (Tokyo, Japan). HPLC-grade water was prepared by purification of tap water, using Elix and Milli-Q academic water purification systems in series (Millipore, Milford, MA, USA).

Phenolic composition of GRE

An Agilent 1200 high performance liquid chromatography (HPLC) system equipped with diode array detector (Agilent, Santa Clara, CA, USA) was used to analyze the extract as described by Beelders et al. (2012a). Briefly, this entailed gradient separation on a 100 $\times$ 4.6 mm 1.8 μ m Agilent Zorbax SB-C18 column protected with an Acquity UPLC in-line filter (Waters, Milford, MA, USA) and a 5.0 μ m Agilent Zorbax SB-C18 guard column, performed at 37 $^{\circ}$ C. The flow rate of the solvent mixture, comprising 2 % (m/v) acetic acid in water and acetonitrile, was 1 ml/min. Stock solutions of the phenolic standards, prepared in dimethylsulfoxide (DMSO), were used to prepare working mixtures of the standards. These were further diluted, using aqueous ascorbic acid (final concentration 10 mg/ml) to stabilize the compounds during analysis. Authentic standards of all compounds, except

quercetin-3-*O*-robinobioside (QROB), were used for preparation of five-point calibration curves. QROB was quantified in rutin equivalents. The extract was reconstituted in deionised water and similarly stabilized against oxidation by diluting with an aqueous ascorbic acid solution. The standard mixtures and reconstituted extract were filtered using 0.22 µm pore-size Millex-GV syringe filter devices (Millipore) prior to HPLC analysis (duplicate injections). Peaks were identified by comparing retention times and UV–Vis spectra with those of authentic standards and by comparison to relative retention times reported by Beelders et al. (2012a).

Determination of glucose uptake by cultured L6 myotubes

Stock cultures of L6 myotubes were maintained in DMEM supplemented with 10 % (v/v) FBS, streptomycin (100 µg/ml), and penicillin G (100 U/ml) (10 % FBS/DMEM) under an atmosphere of 5 % CO₂/95 % humidified air at 37 °C as previously described (Yagasaki et al. 2003). The effect of GRE on glucose uptake was examined according to the procedure previously described (Kawano et al. 2009; Minakawa et al. 2011; Cheong et al. 2014a, b) with slight modifications. Briefly, L6 myoblasts (5×10^4 cells/well) were sub-cultured into Nunc 24-place multiwall plates and grown for 11 days to form myotubes in 0.4 ml of 10 % FBS/DMEM. The medium was replaced every 3 days. The 11-day-old myotubes were kept for 2 h in Krebs-Henseleit buffer (pH 7.4) containing 0.1 % BSA, 10 mM Hepes, and 2 mM sodium pyruvate (KHH buffer). The myotubes were then cultured in KHH buffer containing 11 mM glucose with or without GRE (0–800 µg/ml) or ASP (50 µM) for another 4 h. GRE and ASP were dissolved in KHH buffer. Glucose concentrations in KHH buffer were determined using a glucose assay kit, and the amounts of glucose consumed were calculated from the differences in glucose concentrations before and after culture. This assay system using L6 myotubes is comparable to soleus muscle (Minakawa et al. 2012).

Preparation of plasma membrane from L6 myotubes

L6 myoblasts (5×10^5 cells) were sub-cultured into Nunc 60 mm dishes and grown for 11 days to form

myotubes in 3 ml of 10 % FBS/DMEM while the medium was renewed every 3 days. Following this the 11-day-old myotubes were kept for 2 h in KHH buffer and then cultured in KHH buffer containing 11 mM glucose with or without GRE (350 µg/ml) or ASP (50 µM) added for appropriate time intervals. The plasma membrane fraction of the myotubes, sampled in this manner, was obtained using the method described by Nishiumi and Ashida (2007). Briefly, to prepare the plasma membrane and post-plasma membrane fraction, L6 myotubes were harvested using buffer A (50 mM Tris–HCl (pH 8.0), 0.5 mM DTT, protease inhibitor cocktail and 1 mM Na₃VO₄ supplied by Nacalai Tesque, Inc.) containing 0.1 % (v/v) IGEPAL CA-630 (Sigma-Aldrich Co), and homogenated using a 21-gauge needle. Each homogenate was centrifuged at 1,000×*g* for 10 min at 4 °C. The precipitate was re-suspended in buffer A containing 1.0 % (v/v) IGEPAL CA-630, placed on ice for 1 h with occasional mixing and was centrifuged at 16,000×*g* for 20 min at 4 °C. The supernatant was collected and stored as the plasma membrane (PM) fraction at –80 °C until analyses. The supernatant from the first 1,000×*g* centrifugation was collected and re-centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant obtained was collected and used as a post-plasma membrane (Post-PM) fraction.

Preparation of cell lysate

The 11-day-old myotubes were kept for 2 h in KHH buffer, followed by culturing in KHH buffer containing 11 mM glucose with GRE (350 µg/ml) or ASP (50 µM) for appropriate time intervals. The cells were scraped from the plates into ice-cold RIPA lysis buffer (Nacalai Tesque Inc.) and centrifuged for 20 min at 4 °C and 12,000 × *g*. The supernatant was collected and stored at –80 °C until use.

Western blotting

Protein samples were separated by SDS-PAGE and transferred to a PVDF membrane. The membranes were incubated in a blocking solution, 5 % BSA in Tris-buffered saline (TBS) containing 0.05 % Tween 20, for 2 h, washed three times with TBS containing 0.05 % Tween 20 (TBST) and incubated with primary antibodies overnight at 4 °C. The membranes were then washed three times with TBST and incubated for

2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. Immuno-responsive bands were detected using SuperSignal West pico chemiluminescent substrate. The intensity of each band was analyzed using a lumino image analyzer (Model LAS-3000; Fujifilm, Tokyo, Japan) coupled with image analysis software (Multi Gauge Ver. 3.0; Fujifilm).

Syntheses of advanced glycation end products (AGEs)

AGEs were generated from co-incubation of BSA with either D-glucose (AGE1) or D-glyceraldehyde (AGE2) according to the method of Kume et al. (2005). AGE1 and AGE2 were incubated at 37 °C for 8 and 2 weeks, respectively. BSA alone was incubated at 37 °C for 2 weeks under conditions without any carbohydrates, and used as the control for AGE1 and AGE2. This BSA was designated as CNT.

Measurement of intracellular ROS

Measurement of ROS levels in RIN-5F cells was conducted as described (Son et al. 2013). Briefly, RIN-5F cells derived from rat pancreatic β -cells were maintained in RPMI 1640 supplemented with 10 % (v/v) FBS (10 % FBS RPMI 1640) under an atmosphere of 5 % CO₂/95 % humidified air at 37 °C. The medium was renewed every 3 days. The cells (5×10^5 cells/well) were cultured in Nunc 6-place multiwell plates. After being cultured for 72 h in 2 ml of 10 % FBS/RPMI 1640, the medium in each well was removed. Thereafter, RIN-5F cells received 2 ml of fresh medium (1 % FBS/RPMI 1640) without or with GRE, AGEs and H₂O₂ (Wako Pure Chemical Industries, Ltd.) added for another 3 h. The effect of GRE on oxidative stress was examined by measurement of intracellular ROS based on ROS-mediated conversion of non-fluorescent 2',7'-DCFH-DA into DCFH (Mendis et al. 2007). The intensity of fluorescence reflects enhanced oxidative stress. After the 3 h incubation period, RIN-5F cells were incubated with DCFH-DA (final 5 μ M) in 1 % FBS/RPMI 1640 at 37 °C for 20 min, thereafter DCFH fluorescence of the cells from each well was measured (emission: 530 nm; excitation: 488 nm) using a flow cytometer (EPICS ELITE EPS, Beckman-Coulter, Hialeah, FL, USA).

Cell viability test

The cells (2.5×10^5 cells/well) were cultured in Nunc 6-well multiwell plates for 48 h in 2 ml of 10 % FBS/RPMI 1640. The medium in each well was then removed and replaced with 2 ml of fresh medium (1 % FBS/RPMI 1640) without or with GRE and AGEs added for another 6 h. Total accumulation of viable cells was estimated by hand counting using a hemocytometer after Trypan blue exclusion.

Animals and diets

All animal experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology and were approved by this committee. Male KK-A^y/Ta Jcl and C57BL/6J Jcl mice (3 weeks of age) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Animals were individually housed in stainless-steel cages with wire bottoms in a controlled environment of 22 ± 2 °C, 60 ± 5 % relative humidity and a 12 h light-dark. All the mice were first maintained on a stock CE-2 pellet diet (CLEA Japan, Inc.) for 3 days and thereafter on a basal 20 % casein diet (20C) for 4 days. The composition of the 20C diet was described elsewhere (Minakawa et al. 2011). After preliminary feeding for 1 week, blood was collected from the tail vein at 12:00 after being deprived of their diet at 09:00 but allowed free access to water until blood collection. Blood (10 μ l) was burst in water (40 μ l), 20 % (w/v) trichloroacetic acid aqueous solution (50 μ l) was added, and the test tube containing the mixture was kept in ice-cold water. The mixture was then centrifuged at $13,000 \times g$ and 4 °C for 5 min. The resultant supernatant (10 μ l) was analyzed for glucose concentration using the Glucose CII-Test Kit and measurement of absorbance at 508 nm using a microplate reader. Subsequently, KK-A^y mice (4 weeks of age) were divided into two groups having similar fasting blood glucose levels and body weights (0 week). The KK-A^y mice in each of the two groups were given either the 20C diet as a diabetic control group or the 20C diet supplemented with GRE at 0.3 % (initial 3 weeks) or 0.6 % (subsequent 2 weeks) as a GRE-treated diabetic group for 5 weeks. Water and each diet were freely available, and blood was collected every week at 12:00 to determine the fasting blood glucose levels as described above. At the end of feeding period, blood was collected at 12:00 from the tail vein, followed by exsanguination.

Fig. 1 HPLC chromatogram of a cold water-based green rooibos extract (GRE) at 288 and 350 nm

Peak nr	compound	g compound/100 g powder
1	Phenyl pyruvic acid-2-O-β-D-glucoside (PPAG)	0.36
2	Isoorientin	1.06
3	Orientin	1.07
4	Aspalathin	6.62
5	Quercetin-3-O-robinobioside (QROB)	0.47
6	Vitexin	0.20
7	Hyperoside	0.15
8	Rutin	0.27
9	Isovitexin	0.11
10	Isoquercitrin	0.16
11	Luteolin-7-O-glucoside	0.08
12	Nothofagin	0.48

from the heart under anesthesia with Somnopentyl (Kyoritsu Seiyaku Corporation, Tokyo, Japan). C57BL/6J mice were given the basal 20C diet for 5 weeks as the normal (NOR) group and treated in the same way as KK-A^y mice (CNT, GRE).

Statistical analyses

All data are presented as means $\pm$ standard errors of means (SEM). Multigroup comparisons were carried out by one-way analysis of variance followed by Tukey–Kramer multiple comparisons test. Values of $P < 0.05$ were considered statistically significant.

Results

Phenolic composition of GRE

HPLC chromatograms of GRE, collected at 288 nm (for dihydrochalcones) and 350 nm (for flavonols and

flavones), are depicted in Fig. 1. Quantitative data confirmed that ASP, as expected, was the major flavonoid (6.62 %, dry weight basis) in GRE, followed by its flavone derivatives, orientin (1.07 %) and isoorientin (1.06 %). Lower amounts for its 3-deoxy dihydrochalcone derivative, nothofagin (0.48 %), the phenylpropanoic acid glucoside, phenyl pyruvic acid-2-O-β-glucoside (PPAG, 0.36 %), and the flavonol glycosides, quercetin-3-O-robinobioside (QROB, 0.47 %) and rutin (0.27 %), were present. Small amounts of the flavone derivatives of nothofagin, vitexin and isovitexin, and other flavonol glycosides (≤ 0.2 %) were also present (Fig. 1).

Effect of GRE and ASP on glucose uptake in cultured L6 myotubes

GRE increased glucose uptake in L6 myotubes in a dose-dependent manner, with GRE concentrations of 400–800 $\mu\text{g/ml}$ significantly increasing glucose

Fig. 2 Effect of GRE and aspalathin on glucose uptake, AMPK and Akt phosphorylation, and GLUT4 translocation in L6 myotubes. **a–c** Glucose uptake assay was conducted without or with GRE (0–800 $\mu\text{g/ml}$) or aspalathin (50 μM) and compound C (10 μM). Each value represents the mean $\pm$ SEM for 6 wells. Values not sharing a common letter are significantly different at $P < 0.05$ by Tukey–Kramer multiple comparisons test. **d–f** Western blotting analysis for AMPK and Akt phosphorylation and translocation of GLUT4 to the plasma membrane. Western blotting was conducted with anti-phospho-AMPK, anti-AMPK, anti-phospho-Akt, anti-Akt, anti-GLUT4 and anti- Na^+K^+ -ATPase antibodies. Na^+K^+ -ATPase is a marker enzyme of plasma membrane. *PM* plasma membrane fraction; *Post-PM* post-plasma membrane fraction. The bar diagram in **f** shows the GLUT4/ Na^+K^+ -ATPase ratio of PM fraction to demonstrate the translocation of GLUT4 to the plasma membrane

uptake in the absence of insulin (Fig. 2a). We have already reported that ASP promotes glucose uptake in L6 myotubes at a concentration of 50 μM (Son et al. 2013). To investigate the effect of extract component(s) other than ASP in GRE on glucose uptake in L6 myotubes, we next examined the effect of ASP and GRE. GRE at a concentration of 350 $\mu\text{g/ml}$ in the medium was calculated to contain approximately 50 μM of ASP from its molecular weight and content

in GRE. Both ASP (50 μM) and GRE (350 $\mu\text{g/ml}$) significantly increased glucose uptake in L6 myotubes. AICAR, an AMPK activator, was employed as positive control at a concentration of 1 mM (Fig. 2b). GRE-induced glucose uptake was significantly suppressed by concomitant addition of GRE (350 $\mu\text{g/ml}$) and compound C (10 μM), a competitive inhibitor of AMPK, under the condition that compound C alone exerted no influence on glucose uptake (Fig. 2c).

Fig. 3 Effect of GRE on ROS levels and viability of RIN-5F cells. **a** Basal ROS levels in RIN-5F cells, **b** AGEs-induced ROS level and **c** H₂O₂-induced ROS level in RIN-5F cells. **d** Viability of RIN-5F cells without or with AGE2 and GRE. Each value represents the mean $\pm$ SEM for 3 wells. Values not sharing a common letter are significantly different at $P < 0.05$ by Tukey–Kramer multiple comparisons test

These results indicate, at least partly, an involvement of AMPK in promotion of glucose uptake by GRE in L6 myotubes.

Effect of GRE and ASP on phosphorylation of AMPK and Akt in cultured L6 myotubes and translocation of GLUT4 to plasma membrane

L6 myotubes were treated with either 50 μM ASP, 350 μg/ml GRE, or 1 mM AICAR for 240 min. All increased the phosphorylation ratio of AMPK (p-AMPK/AMPK), but GRE more notably promoted AMPK phosphorylation than did ASP alone (Fig. 2d). GRE also increased the phosphorylation of Akt (p-Akt/Akt), whereas ASP alone had no significant effect (Fig. 2e).

These results prompted us to determine the effect of GRE on translocation of GLUT4 to the plasma membrane. L6 myotubes, when treated with GRE (350 μg/ml) for 240 min, showed translocation of GLUT4 to the plasma membrane (Fig. 2f).

Protective effect of GRE on oxidative stress in cultured RIN-5F cells

GRE significantly and dose-dependently reduced the basal ROS levels in cultured RIN-5F cells (Fig. 3a). To examine the protective effect of GRE against oxidative stress, we determined the effect of GRE on AGEs-induced ROS levels in RIN-5F cells. AGE1 and AGE2, representing BSA conjugated with glucose and glyceraldehyde, respectively, significantly increased the intracellular ROS levels when compared with that of the control (CNT; BSA alone). GRE (50 μg/ml) significantly reduced the AGEs-induced ROS levels (Fig. 3b). Likewise, the addition of H₂O₂ (5 μM) to culture media increased the intracellular ROS levels compared with that of CNT, whereas GRE (50 μg/ml) significantly reduced the H₂O₂-induced increase in ROS levels (Fig. 3c). These results suggest that GRE is capable to protect cultured RIN-5F cells from the oxidative stress by its ROS scavenging ability.

Fig. 4 Effect of GRE feeding on fasting blood glucose levels of KK- A^y mice. During 5 weeks of GRE feeding, fasting blood glucose levels were measured once a week after mice were fasted for 3 h before blood collection. C57BL/6J mice were given the basal 20C diet for 5 weeks as the normal (NOR) group and treated in the same way as KK- A^y mice (CNT, GRE). Each value represents the mean $\pm$ SEM (NOR: $n = 7$, CNT: $n = 6$, GRE: $n = 5$). Values not sharing a common letter are significantly different at $P < 0.05$ by Tukey–Kramer multiple comparisons test

Furthermore, treatment of cultured RIN-5F cells with AGE2 for 6 h induced cell death, but GRE (50 $\mu\text{g/ml}$) was effective ($P < 0.05$) in suppressing AGE2-induced damage in RIN-5F cells (Fig. 3d).

Effect of GRE on fasting blood glucose levels in obese diabetic mice

To study the *in vivo* effect of GRE, type 2 diabetes model KK- A^y mice were fed the basal 20C or 20C diet supplemented with 0.3 % (initial 3 weeks) or 0.6 % (following 2 weeks) of GRE for 5 weeks. Because we could not obtain any significant glucose-lowering effect at 0.3 % GRE in the diet for 3 weeks, we increased the dose of GRE to 0.6 % for the next 2 weeks. The fasting blood glucose levels of the diabetic control mice (CNT) were significantly higher than that of normal mice (NOR) (Fig. 4). However, 2 weeks after feeding KK- A^y mice with GRE, its suppressive effect on fasting blood glucose levels became evident. This glucose-lowering effect was significant after treatment with GRE for 4 weeks, while the lowering effect was not significant 5 weeks

after GRE feeding, being probably due to further decline in the blood glucose levels of corresponding diabetic control KK- A^y mice (CNT) that were lower than those obtained 4 weeks after 20C feeding (Fig. 4). There were no differences in body weight gain and food intake between diabetic control and GRE-treated KK- A^y mice during the feeding period (data not shown).

Discussion

In our previous study, ASP was demonstrated to suppress the increase in fasting blood glucose levels, improve impaired glucose tolerance in type 2 diabetic model db/db mice (Kawano et al. 2009) and promote glucose uptake through GLUT4 translocation to the plasma membrane via AMPK activation in L6 myocytes (Son et al. 2013). ASP also reduced the AGEs-induced ROS in RIN-5F cells and showed antidiabetic effect in ob/ob mice (Son et al. 2013). In the present study, we determined the effect of GRE on L6 myotubes, pancreatic β -cells and hyperglycemia in type 2 diabetic model KK- A^y mice. We also compared *in vitro* the effect of GRE with that of ASP. For this comparison, the concentration of GRE in the medium was adjusted so that the amount of ASP derived from GRE was equal to that of pure ASP in the medium, in an attempt to elucidate the effect of other extract constituents. A complete characterization of the water soluble fraction of unfermented rooibos has not yet been attempted, therefore compositional analysis of GRE focused on its major monomeric phenolic compounds that could be quantified. A range of minor phenolic compounds have previously been demonstrated to be present in unfermented rooibos extract (Beelders et al. 2012a, b). Compounds tentatively identified included a C-5'-hexosyl derivative and flavanone derivatives of aspalathin, as well as a cyclic dihydrochalcone, aspalalinin and luteolin-di-C-glycosides (Beelders et al. 2012b). Rooibos also contain the antidiabetic inositol derivative, pinitol (Rabe et al. 1994; Hernández-Mijares et al. 2013).

GRE significantly promoted glucose uptake in cultured L6 myotubes and its effect was reduced by treatment with the AMPK inhibitor, compound C. This result shows that GRE increased glucose uptake

through the AMPK pathway. AMPK is known to play a role in improving insulin insensitivity by direct stimulation of glucose uptake in muscle, independently of the insulin signaling pathway (Iglesias et al. 2002; Fisher et al. 2002). In the present study, GRE activated Akt as well as AMPK. Akt is known to participate in the insulin signaling pathway and to promote the translocation of GLUT4 to the plasma membrane (Huang and Czech 2007). Compound C significantly suppressed GRE-induced promotion of glucose uptake. However, glucose uptake was still slightly higher in GRE- and compound C-treated myotubes than in control ones (Fig. 2c), suggesting that a subtle contribution of Akt activation toward promoted glucose uptake by GRE may not be excluded. Further studies are required to clarify this aspect. Unlike GRE, ASP did not promote the phosphorylation of Akt but only increased that of AMPK (Son et al. 2013). By comparing the effects of GRE with ASP at the same ASP concentration (50 μ M) on L6 myocytes, we found that GRE appeared to be more effective on glucose uptake and AMPK activation than ASP. GRE contained other polyphenols that, in addition to ASP, could contribute to this effect. Rutin was shown to attenuate induced glucotoxicity in pancreatic β -cells, amongst others by activating AMPK (Cai and Lin 2009). Rutin was also demonstrated to enhance the glucose lowering effect of ASP in STZ-induced diabetic rats after acute oral administration (Muller et al. 2012). Isoorientin demonstrated significant hypoglycemic effect in STZ-induced diabetic rats (Sezik et al. 2005). In obese insulin-resistant rats, oral administration of PPAG lowered fasting glucose concentrations and improved oral glucose tolerance values (Muller et al. 2013). Further studies concerning the effects of these and other components in GRE on AMPK and Akt phosphorylation and their interactions in cultured muscle cells and muscle tissues of diabetic model animals are needed to elucidate the precise mechanisms for the observed GRE effect.

In the diabetes state, the glycation reaction is observed in various tissues and organs, and many kinds of glycated proteins such as AGEs are formed. AGEs are well known to produce ROS by binding to their receptors, thus playing a significant role in the development of diabetic complications (Wautier and Guillausseau 2001; Fiorentino et al. 2013). The serum level of AGEs is reportedly associated with insulin

resistance even in non-obese, non-diabetic subjects (Tan et al. 2011). Hence, suppressing the AGEs-induced ROS contributes toward protection of the development of diabetes and its complications. Robertson et al. (2007) hypothesized that ancillary treatment with antioxidants may improve outcomes of standard therapy of type 2 diabetes in humans. For this reason, GRE containing high levels of free radical scavengers was also evaluated for its ability to reduce the AGEs-induced and H_2O_2 -induced oxidative stress in RIN-5F cells. This effect was then demonstrated for GRE at a concentration of 50 μ g/ml, equivalent to approximately 7.3 μ M of ASP, confirming its antioxidative potential and protective role as ROS scavenger in pancreatic β -cells. In our previous study, ASP also reduced AGEs-induced ROS in RIN-5F cells at the concentrations of 25–100 μ M (Son et al. 2013). In this study, we found that GRE protected RIN-5F cells from AGEs-induced ROS at lower ASP-equivalent concentrations than those of ASP treatment alone. GRE also suppressed AGEs-induced cell death in RIN-5F cells. From these results, GRE is suggested to be more effective than ASP alone in protecting pancreatic β -cells from oxidative stress probably due to its diverse constituents.

GRE was demonstrated to suppress the elevation in the fasting blood glucose level in type 2 diabetic model KK- A^y mice. The fasting blood glucose levels in the diabetic control KK- A^y mice (CNT) were lower at 4th and 5th weeks of feeding than at 3rd week of feeding. One of the reasons for this phenomenon might be attributable to increased excretion of glucose into urine at 4th and 5th weeks of feeding as compared with its excretion at 3rd week of feeding (Ha et al. 2012; Cheong et al. 2014b), this being one of the reasons why the glucose-lowering effect was not significant 5 weeks after GRE treatment (Fig. 4). Food intake during the experimental period of 5 weeks was similar between diabetic control and GRE-fed KK- A^y mice, indicating that the suppressive effect of GRE on the increase in fasting blood glucose concentrations was not due to reduced food consumption but due to its pharmacological action. These results serve as a further confirmation of the antidiabetic potential of GRE *in vivo*. It is necessary to analyze common parameters *in vivo* such as levels of AGEs, AMPK phosphorylation and GLUT4 translocation in the skeletal muscle of KK- A^y mice to connect to the results on those obtained *in vitro* in the present study.

Further intensive studies are required to clarify the precise mechanisms for antidiabetic action of GRE and elucidate differences and/or similarities in actions of GRE and ASP.

In summary, GRE promoted glucose uptake through translocation of GLUT4 to the plasma membrane via activation of both AMPK and Akt in L6 myotubes. GRE scavenged AGEs- and H₂O₂-induced ROS in RIN-5F cells and it was more effective than ASP alone at the equivalent dose in GRE. Furthermore, GRE reduced the increases in fasting blood glucose levels in type 2 diabetic model KK-A^y mice. Combined these results strongly suggest antidiabetic potential of GRE.

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Conflict of interest The authors declare that they have no conflict of interest.

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