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Screening plant derived dietary phenolic compounds for bioactivity related to cardiovascular disease

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ABSTRACT

The potential health benefits of phenolic acids found in food and beverages has been suggested from a number of large population studies. However, the mechanism of how these compounds may exert biological effects is less well established. It is also now recognised that many complex polyphenols in the diet are metabolised to simple phenolic acids which can be taken up in the circulation. In this paper a number of selected phenolic compounds have been tested for their bioactivity in two cell culture models. The expression and activity of endothelial nitric oxide synthase (eNOS) in human aortic endothelial cells and the uptake of glucose in muscle cells. Our data indicate that while none of the compounds tested had a significant effect on eNOS expression or activation in endothelial cells, several of the compounds increased glucose uptake in muscle cells. These compounds also enhanced the translocation of the glucose transporter GLUT4 to the plasma membrane, which may explain the observed increase in cellular glucose uptake. These results indicate that simple cell culture models may be useful to help understand the bioactivity of phenolic compounds in relation to cardiovascular protection.

1. Introduction

Initial interest in the possible role of dietary plant components protecting against cardiovascular disease (CVD) was sparked by pivotal human intervention studies in the early 1980's showing that a vegetarian diet could lower blood pressure [1]. Population studies in the 1990's also started to show that dietary flavonoids were associated with reduced risk of CVD [2]. During this time there was growing evidence that oxidative modification of lipoproteins, in particular low density lipoproteins (LDL), played an important role in atherogenesis [3]. Early reports that beverages such as red wine that contained high concentrations of polyphenolic substances could inhibit LDL oxidation stimulated huge interest in this field [4]. Over the past three decades our understanding of the role of diet derived polyphenolic compounds in disease prevention has grown. They were initially considered as simple antioxidants, but they and their in vivo metabolites, are now seen as signalling molecules [5].

Some beverages such as coffee [6,7] and honey derived products such as propolis [7], contain a range of simple phenolic acids and while the chemistry of these compounds in not particularly interesting, their bioactivity is potentially very interesting [8]. Coffee consumption has been associated with reduced risk of developing type 2 diabetes [9] and while the exact mechanism involved remains unknown, the caffeic acid derivatives in coffee have been shown to improve vascular function and glucose metabolism [10]. Propolis is rich in phenolic compounds and there is evidence that these compounds can promote glucose uptake and may help prevent hyperglycemia [11].

We have been interested in developing convenient bioassays that may help understand the potential bioactivity of phenolic acids in terms of cardiovascular protection. Major risk factors for CVD include endothelial dysfunction, which is characterized by poor bioavailability of nitric oxide (NO·), an important regular of blood vessel function. Most NO· is produced by endothelial nitric oxide synthase (eNOS), so the expression and activation of this enzyme is important in vascular disease. Similarly, high blood glucose (hyperglycaemia) is characteristic of the development of diabetes, which can lead to endothelial dysfunction and in turn is a major risk factor for CVD. Regulation of glucose metabolism is dependent on the uptake of glucose into cells via specific transporters that must be translocated to the cell membrane in order to move glucose into the cell. Insulin is a major physiological stimulator of glucose uptake via glucose transporter 4 (GLUT4). Certain factors such as a high calorie diet can cause a decrease in glucose transporter 4

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(GLUT 4) expression and/or translocation and insulin resistance leading to hyperglycemia [12]. In this report we have focussed on the bioactivity of phenolic acid compounds in two model cell culture systems; these are, the expression and phosphorylation of eNOS in cultured human aortic endothelial cells and, glucose uptake and GLUT4 translocation in skeletal muscle cells. In an attempt to understand the potential signalling pathways which may be altered by phenolic acid compounds we examined the expression and phosphorylation of adenosine monophosphate-activated protein kinase (AMPK), a master regulator of cell metabolism [13] and AKT a protein kinase that plays a key role in glucose metabolism and endothelial function [14]. Downstream targets of AMPK include enzymes of glucose and lipid metabolism, mitochondrial enzymes and eNOS. Activation of AMPK can prevent oxidative stress-induced vascular dysfunction via increased phosphorylation and activation of eNOS [15]. We have also shown that quercetin and its in vivo metabolites can improve vessel function by inducing eNOS via phosphorylation of AMPK in serum starved endothelial cells [16].

This study reports the bioactivity of several phenolic acids and the flavonoid quercetin as a reference compound. Our data indicate that some of these compounds have activity that may help explain the cardio protective effects of dietary phenolic compounds.

2. Experimental methods

2.1. Materials

Dulbeco's Minimum essential medium (DMEM), was obtained from Nissui Pharmaceutical (Tokyo, Japan). 2-deoxyglucose (2DG), 7-hydroxy-10-oxy-phenoxazin-3-one (resazurin), was obtained from Sigma, (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase (G6PDH), diaphorase, and beta-nicotinamide adenine dinucleotide phosphate (NADP) + were from OrientalYeast (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Antibodies for AMPK, AKT and phosphorylated eNOS (peNOS, Ser1177) came from Cell Signalling (Danvers, MA, USA) and were used at 1:500 to 1:1000 dilutions. eNOS antibody was from Millipore and used at 1:500 to 1:1000 dilution. GLUT4 antibody (#2213) was from Cell Signalling and used at 1:5000 dilution. The compounds used for testing were obtained as pure standards from Sigma Aldrich (USA) Chlorogenic acid (**1**), Ferulic acid (**2**), Protocatechuic acid (**3**), Dihydroferulic acid (**4**), Quercetin (**5**) and Gallic acid (**6**), Fig. 1.

2.2. Muscle cell culture for glucose uptake experiments

Rat L6 skeletal muscle cell were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. After the cells have reached confluency, differentiation was induced by replacing the growth medium with DMEM supplemented with 2% FBS. L6 myotubes were cultured and differentiated on a 96-well plate and serum-starved for 18 h in DMEM containing 0.2% (w/v) BSA at 37 °C. L6 myotubes were treated with the compounds at 0.1, 1.0, and 10 μM for 1 and 4 h in 0.2% (w/v) BSA/DMEM. As a positive control, the cells were treated with 100 nM insulin for 1 and 4 h. DMSO was used as a vehicle control (final concentration was 0.1%). The treated cells were further incubated with 1 mM 2DG for 20 min in Krebs–Ringer HEPES buffer (KRH; 50 mM HEPES, pH 7.4, 137 mM sodium chloride, 4.8 mM potassium chloride, 1.85 mM calcium chloride and 1.3 mM magnesium sulfate) containing 0.1% (w/v) BSA. 2-DG uptake was determined by an enzymatic reaction as described previously (Yamamoto N, et al., CurrProtocPharmacol. 2015;71:12.14.1-26.).

2.3. GLUT4 translocation experiments

GLUT 4 translocation experiments were carried out as previously described. Briefly, L6 myotubes were treated with the compounds (1 and 10 μM), insulin (100 nmol/L) or DMSO as a vehicle control for 15 min. The cells were washed twice with ice-cold Krebs-ringer-HEPES buffer, homogenized in buffer A [50 mmol/L Tris, pH 8.0, 0.1% (v/v) Nonidet P-40, 0.5 mmol/L DTT, protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and phosphatase inhibitors (10 mmol/L NaF and 1 mmol/L Na3VO4)] using a hand-held microtube homogenizer and passed through a 27-gauge syringe needle five times. Aliquot of the homogenate was mixed with RIPA buffer [10 mmol/L Tris, pH 8.0, 150 mmol/L sodium chloride, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, and 0.5 mmol/L DTT] containing the same protease and phosphatase inhibitors and incubated on ice for 60 min with occasional mixing. This mixture was centrifugated at 16,000 × *g* for 20 min at 4 °C, and obtained supernatant was referred to as the cell lysate. The remainder of the homogenate was centrifuged at 900 × *g* for 10 min at 4 °C. The resulting pellet was suspended in buffer A and centrifuged under the same conditions. The precipitate was resuspended in buffer A containing 1% (v/v) Nonidet P-40 and the same protease and phosphatase inhibitors, stood on ice for 60 min with occasional mixing, and centrifuged at 16,000 × *g* for 20 min at 4 °C. The obtained supernatant was used as the plasma membrane. Proteins in the plasma membrane and cell lysate fractions of myotubes were separated by SDS-polyacrylamide gels and transferred to the polyvinylidene difluoride membranes. After blocking with commercial Blocking one solution (Nacalai Tesque, Kyoto Japan), the membrane was incubated with the specified primary antibody for GLUT4 overnight at 4 °C, followed by the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins bands were visualized using ImmunoStar® LD (Wako) and detected with a light-Capture II (ATTO Corp., Tokyo, Japan). The density of specific band was analyzed using ImageJ image analysis software (National Institutes of Health, Bethesda, MD).

2.4. Endothelial cell culture

Human aortic endothelial cells (HAECs) (Lonza Pty Ltd., Australia) were maintained in commercially available EBM-2 media (Lonza Pty, Australia). Cells were grown in T-75 flasks and passaged every 3–4 days as necessary. All experiments were performed on confluent cells between passages 4–10 in 6-well culture plates. Prior to experiments, cells were serum-starved for 6 h prior to treatment with test compounds or vehicle (0.1% DMSO) for 15 min or 6 h. Cell lysates and culture media were collected and stored at − 80 °C until analysis.

2.5. eNOS expression and phosphorylation

To determine protein expression following treatment, cells were collected into 200 μL Laemmli's [2 ×] sample loading buffer (Bio-Rad, Australia), with 5% β-mercaptoethanol and phosphatase inhibitor cocktail (PhosSTOP, Roche Diagnostics Australia). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels, transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, USA) overnight and the membranes incubated with primary antibody overnight at 4 °C. After washing and incubation with HRP-conjugated secondary antibody for 1 h at room temperature, protein bands were visualized using ECL reagents (GE Healthcare Life Sciences, USA) on a FluorChem FC2 (Alpha Innotech, USA). Protein bands were analyzed by densitometry using the FluorChem software and normalized to Actin. Antibodies against eNOS and p-eNOS (Ser1177) were purchased from Cell Signalling Technology, USA.

2.6. NOx analysis

NOx (S-nitrosothiols, nitrite and nitroso species) in cell culture media were quantified by reductive denitrosation of samples using a