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Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3-L1 adipocytes

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Abstract

Cinnamon improves glucose and lipid profiles of people with type 2 diabetes. Water-soluble cinnamon extract (CE) and HPLC-purified cinnamon polyphenols (CP) with doubly linked procyanidin type-A polymers display insulin-like activity. The objective of this study was to investigate the effects of cinnamon on the protein and mRNA levels of insulin receptor (IR), glucose transporter 4 (GLUT4), and tristetraprolin (TTP/ZFP36) in mouse 3T3-L1 adipocytes. Immunoblotting showed that CP increased IRβ levels and that both CE and CP increased GLUT4 and TTP levels in the adipocytes. Quantitative real-time PCR indicated that CE (100 μg/ml) rapidly increased TTP mRNA levels by approximately 6-fold in the adipocytes. CE at higher concentrations decreased IRβ protein and IR mRNA levels, and its effect on GLUT4 mRNA levels exhibited a biphasic pattern in the adipocytes. These results suggest that cinnamon exhibits the potential to increase the amount of proteins involved in insulin signaling, glucose transport, and anti-inflammatory/anti-angiogenesis response. Published by Elsevier Inc.

Keywords: Adipocytes; Angiogenesis; Cinnamon extract; Diabetes; Glucose transporter; Inflammation; Insulin receptor; Obesity; Polyphenol; Tristetraprolin

Diabetes has been a subject of extensive research, but the prevention and control of type 2 diabetes mellitus (type 2 DM)[1] has not been resolved. Diet has been shown to play an important role in the development of type 2 DM, and the diets commonly consumed in the United States and other developed countries appear to increase the incidence of diabetes [1]. The higher incidences of diabetes in the US are probably due in part to a combination of higher content of refined sugar and fat and lower intake of traditional herbs, spices, and other plant products. For the majority of people in developing countries, drug treatment for diabetes is not feasible and alternative and inexpensive therapies need to be evaluated.

Plants have been used for the treatment of diabetes since 1550 BC [2]. Plants are important for the prevention and control of type 2 DM, especially for people with elevated levels of blood glucose and glucose intolerance who have a greater risk of developing diabetes. Plant seeds, fruits, leaves, and bark contain polyphenols. These compounds are the end products of the flavonoid biosynthetic pathway in plants and are used by plants for the protection against predators [3]. Plant polyphenols are also widely present in the diet [4] and are important for human health [5].

Common spices (cinnamon, cloves, turmeric, and bay leaves) and tea display insulin-like activity *in vitro* [6,7]. We have identified polyphenolic polymers from an aqueous extract of commercial cinnamon that increase glucose metabolism several fold in an epididymal fat cell assay [8]. These cinnamon polyphenols (CP) with doubly linked procyanidin type-A polymers appear to be unique for their insulin-like

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[1] *Abbreviations used:* ARE, AU-rich element; CE, cinnamon extract; CP, cinnamon polyphenols; DMEM, Dulbecco's modified Eagle's medium; DMEM+, DMEM plus 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine; GLUT4, glucose transporter 4; IR, insulin receptor; TTP, tristetraprolin.

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activity, because other cinnamon compounds display little or no such activity [8]. In addition, none of the other 50 plant extracts tested displayed activity equal to that of cinnamon [7].

Strong evidence suggests that CP exhibit insulin-like activity in cells, animals and people with type 2 diabetes. First, a water-soluble cinnamon extract (CE), like insulin, increases the activity of autophosphorylation of the insulin receptor β (IRβ) and decreases the activity of tyrosine phosphatase *in vitro* [9]. Second, CP, like insulin, stimulate glucose uptake and glycogen biosynthesis, activate glycogen synthase, and inhibit glycogen synthase kinase-3β [10]. Third, CE potentiates *in vivo* insulin-regulated glucose utilization via increasing glucose uptake, and prevents insulin resistance induced by a high-fructose diet in rats [11,12]. It also decreases glucose and increases insulin in blood of rats fed diets containing CE [13] and decreases blood pressure [14]. Finally, cinnamon was shown to decrease the levels of glucose, triglycerides, and LDL cholesterol in people with type 2 diabetes [15]. A recent study involving postmenopausal patients with type 2 diabetes under good control for diabetes (mean HbA1c of 7.1–7.4%) did not respond to cinnamon [16]. It is not clear if this lack of a response is due to selection of patients, level of control, oral hypoglycemic agents, diet or type of cinnamon used.

Cinnamon polyphenols may have additional benefits for human health. First, CE has the ability to inhibit cancer cell proliferation by altering the cell cycle pattern in three myeloid cell lines (Jurkat, Wurzburg, and U937) [17]. Second, cinnamon bark was reported to have antioxidant effects by increasing the activities of antioxidant enzymes including glutathione *S*-transferase, superoxide dismutase, and catalase in rat livers and hearts [18]. Third, it was reported that CE has anti-ulcerogenic activity by preventing the occurrence of stress ulcers under cold exposure or water-immersion-stress in rats [19]. Finally, it is possible that CP may have anti-inflammatory properties because insulin induces the mRNA levels of the anti-inflammatory protein tristetraprolin (TTP) in mouse cells [20].

Cell cultures have been used as model systems in the studies of the mechanisms of plant polyphenols in animal and human health [5]. Polyphenols have been shown to alter signal transduction pathways in cultured cells [5]. To understand the molecular basis of insulin-like activity and explore additional benefits of cinnamon polyphenols, we investigated the effects of CE and CP on the regulation of IRβ, glucose transporter 4 (GLUT4) and TTP in mouse 3T3-L1 adipocytes. These three proteins are involved in the insulin signaling transduction pathway that functions in insulin receptor substrate activation [21], insulin-regulated glucose transport [22], and anti-inflammatory responses [23], respectively.

Materials and methods

Cinnamon extract and polyphenols

Water-soluble CE was prepared as described previously [8] with modifications. Briefly, ground cinnamon (*Cinnamomum burmannii*) was suspended in 0.1 N acetic acid. The suspension was autoclaved for 15 min at 15 psi and the supernatant was mixed with four volumes of absolute ethanol and refrigerated overnight. The mixture was filtered through glass wool and then Whatman No. 1 filter paper. The ethanol was removed by rotoevaporation and the remaining solution was freeze-dried. The dried CE was reconstituted at 100 mg/ml in 100% dimethylsulfoxide (DMSO) and diluted with deionized water before being added to the culture medium. CE powder was stored at room temperature and the reconstituted samples were kept at −20 °C for long-term storage and at 4 °C for short-time storage.

Cinnamon polyphenols were purified from CE by high performance liquid chromatography (HPLC) [8]. Briefly, CE was filtered through a 0.45 μm filter before being injected onto a Symmetry Prep C_{18} column (7.8 × 300 mm) and separated by reverse phase HPLC at a flow rate of 4 ml/min using a two-step program: (1) 0–50 min, 92% of 0.05 N HAc and 8% acetonitrile (Fractions 1–6); (2) 51–58 min, gradient to 100% acetonitrile (Fraction 7). Cinnamon polyphenol fractions from HPLC were designated as CP1A, CP1B, CP2, CP3, CP4, CP5, CP6, and CP7. Fractions 2, 4, and 6 have been characterized [8]. CP2 is a procyanidin trimer (M_r 864 Da) (Fig. 1a). CP4 is a tetramer (M_r 1152 Da). CP6 is a trimer with the same molecular mass as CP2 (M_r 864 Da). CP7 is a mixture of monomer (M_r 288 Da) plus other oligomers as determined by mass spectrometry analyses [8]. CP3 and CP5 contained mixtures of trimers and tetramers and the exact identities of CP1A and CP1B were not determined. The fractions were collected and acetonitrile removed by rotoevaporation and freeze-dried. The freeze-dried samples were reconstituted at 10 mg/ml in 100% DMSO and stored as described above for the storage of CE.

Cell culture

Mouse 3T3-L1 fibroblasts (American Type Culture Collection) were maintained at 37 °C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/l (25 mM) glucose (Gibco BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (DMEM+). Adipocyte induction was as described [10]. Mouse 3T3-L1 fibroblasts (about 0.2 million cells/2-ml medium/well) were grown in 6-well plates under the same conditions for 48–60 h and the medium was replaced with fresh DMEM+. After incubation for another 48–60 h, the medium was replaced with differentiation medium containing DMEM+, 1 μg/ml of the recombinant human insulin expressed in yeast (Sigma Chemical Co, St. Louis, MO), 0.25 μM dexamethasone (Sigma), and 250 μM 1-isobutyl-3-methylxanthine (IBMX) (Sigma). Following incubation for 48–60 h, the differentiation medium was replaced with DMEM+ containing only 1 μg/ml of insulin. After incubation for additional 48–60 h, the medium was replaced with DMEM+ and the cells were grown for an additional 4–6 days. Microscopic observation indicated that approximately 80–90% of the cells accumulated lipid drops (indication of differentiation from preadipocytes to adipocytes) (Fig. 1b). The cells were then serum-starved in DMEM without any supplementation for 3–4 h before various chemicals and the vehicle control at its highest concentration were added to the medium for various times as indicated in the "Result" section and the figure captions. Two to four independent experiments were performed. Cellular extract was prepared as described below.

Mouse macrophage RAW264.7 cells (American Type Culture Collection) were cultured in Eagle's minimum essential medium (Gibco BRL), and were treated with 0.1 μg/ml lipopolysaccharide (LPS) (Sigma) for 2 h [24]. The induced TTP was used as a positive control in SDS–PAGE.

Cell extracts

Cell extracts were prepared as described [24] with modifications. Briefly, after washing twice with 0.9% NaCl, 100–150 μl of lysis buffer containing 50 mM NaH_2PO_4, pH 7.6, 250 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.2% (v/v) of protease inhibitor cocktails (104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64) (Sigma) were added to each well. The cells were scraped and transferred into microfuge tubes and left on ice for about 30 min before being centrifuged at 10,000*g* for 10 min at 4 °C. The 10,000*g* supernatant was stored at −20 °C.