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Isolation and Characterization of Polyphenol Type-A Polymers from Cinnamon with Insulin-like Biological Activity

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The causes and control of type 2 diabetes mellitus are not clear, but there is strong evidence that dietary factors are involved in its regulation and prevention. We have shown that extracts from cinnamon enhance the activity of insulin. The objective of this study was to isolate and characterize insulin-enhancing complexes from cinnamon that may be involved in the alleviation or possible prevention and control of glucose intolerance and diabetes. Water-soluble polyphenol polymers from cinnamon that increase insulin-dependent in vitro glucose metabolism roughly 20-fold and display antioxidant activity were isolated and characterized by nuclear magnetic resonance and mass spectroscopy. The polymers were composed of monomeric units with a molecular mass of 288. Two trimers with a molecular mass of 864 and a tetramer with a mass of 1152 were isolated. Their protonated molecular masses indicated that they are A type doubly linked procyanidin oligomers of the catechins and/or epicatechins. These polyphenolic polymers found in cinnamon may function as antioxidants, potentiate insulin action, and may be beneficial in the control of glucose intolerance and diabetes.

KEYWORDS: Glucose; insulin; diabetes; cinnamon; polyphenols; spice

INTRODUCTION

Despite extensive diabetes research, the prevention and control of type 2 diabetes mellitus (type 2 DM) remain unclear. Diet has been shown to play a definite role in the onset of type 2 DM, and the diets commonly consumed in the United States and other westernized countries appear to increase the incidence of diabetes (*1*). The high refined sugar and high fat content of US diets are likely to be partly responsible, but the low intake of traditional herbs, spices, and other plant products may also be involved. The recommended use of plants in the treatment of diabetes dates back to approximately 1550 BCE (*2*). For the majority of the world population, drug treatment for diabetes is not feasible and alternative treatments need to be evaluated. Plants are important not only for the control of type 2 DM but also for its prevention, especially for people with elevated levels of blood glucose and glucose intolerance who have a greater risk of developing diabetes.

Common spices such as cinnamon, cloves, and bay leaves display insulin potentiating activity in vitro (*3*). It was thought that these spices might also have high chromium (Cr) concentrations, because biologically active forms of Cr potentiate insulin activity (*4*). However, there are no correlations between total Cr concentrations and insulin potentiating activity in these plant products (*3*). Only a small portion of the total Cr in biological systems is associated with insulin potentiating activity.

In addition to improving cellular glucose metabolism, cinnamon may provide additional benefits for persons with diabetes through its antioxidant activity. Cinnamon, in addition to cloves, cumin, curcumin, many mint family plants, and others that are usually high in flavonoids, which are potent antioxidants, may be synergistic with vitamins and trace elements (*5*−*8*). Dried ground and fresh spices were found to be highly effective at preventing lipid peroxidation of cooked ground fish. The order of effectiveness for dried spices was cloves > cinnamon > cumin ≥ black pepper ≥ fennel = fenugreek (*9*). Specific antioxidant phytochemicals that have been identified in cinnamon include epicatechin, camphene, eugenol, gamma-terpinene, phenol, salicylic acid, and tannins (*8*).

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From an aqueous extract of commercial cinnamon, we have identified polyphenolic polymers that increase glucose metabolism roughly 20-fold in vitro in the epididymal fat cell assay (*10*). These appear to be rather unique, because other cinnamon or similar compounds display little or no biological activity. Additionally, approximately 50 plant extracts have also been investigated in this assay, and none have shown activity equal to that of cinnamon (*11*).

MATERIALS AND METHODS:

Three verified samples of cinnamon were tested, including Korintji lauraceae; Microbial Identification Index (MIDI) class; Korintji cassia; botanical class, *Cinnamomum burmannii* (Nees) Blume; a cinnamon of Chinese origin, MIDI class Tung Hing, botanical class, *C. cassia* Blume (Lauraceae); and one of Vietnamese origin, botanical class *C. loureirii* Nees, (Lauraceae); which were obtained as a generous gift from Dr. Carolyn Fisher, McCormick & Company Incorporated, Baltimore, MD. Samples were verified using a database that utilizes a fingerprint matching of the volatile oil components. Other ground commercial cinnamons tested were *C. verum* (Ceylon cinnamon or "canela", bulk bark) *C. loureinii* (Vietnamese cinnamon) and *C. burmannii* (Korintje cinnamon from Sumatra) (Penzey's House of Spices, Muskego, WI) as well as a *C. cassia* specimen in bulk bark from China. The cinnamon used for the purification and characterization was primarily *C. burmanni*.

Insulin enhancing biological activity was measured using the epididymal fat cell assay (*10*, *11*). Briefly, 0.43 μCi [U-^{14}C]-glucose, 72 μg glucose, and adipocytes were incubated with insulin and/or aqueous extracts of cinnamon or its components in a final reaction volume of 2 mL of Krebs-Ringer phosphate buffer, pH 7.1. Quantitation of $^{14}CO_2$ release by the cells was done using benzethonium hydroxide (Sigma-Aldrich, St. Louis, MO) as a trapping agent, which is a replacement for hyamine hydroxide. Similar results were obtained by trapping $^{14}CO_2$ and measuring ^{14}C incorporation into lipids using Dole's solution (800 mL of 2-propanol, 200 mL of heptane, and 20 mL of 1 N sulfuric acid). The insulin activity ratio was calculated by dividing the basal cpm of $^{14}CO_2$ released by the cells into those of the activity due to aqueous extracts of cinnamon or its components.

For the purification of the active components of cinnamon, 5 g of cinnamon was suspended in 100 mL of 0.1 N acetic acid and autoclaved for 15 min at 15 psi. The supernatant was removed, 4 volumes of absolute ethanol were added to the supernatant, and the sample was stored at 4 °C overnight. The sample was filtered using Whatman #40 filter paper. The sample was added to an LH-20 column (5 × 15 cm) (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with absolute ethanol and washed with 600 mL of absolute ethanol. The sample was then eluted with 50% acetonitrile and water with a final concentration of 0.1N acetic acid. Fractions with insulin-enhancing activity were collected and concentrated by rotoevaporation and purified using high performance liquid chromatography (HPLC). Samples were injected onto a 7.8 × 300 mm, 7 μL, SymmetryPrep C18, column (Waters Corp., Milford, MA), equilibrated with 90% 0.05 N acetic acid and 10% acetonitrile at a flow rate of 4 mL/min using a Waters HPLC chromatography system with Millennium 2100 software and a Waters 996 ultraviolet absorbance detector.

The production of reactive oxygen species was determined using whole blood samples from rats (*12*). The molecular probe, 2′7′-dichlorodihydrofluorescein diacetate, 10 μM, was loaded into the platelets for 10 min, followed by a 5 min exposure to insulin potentiating polyphenolic polymers or vehicle control. Collagen at 20 μg/mL was added and platelets in the samples were monitored for increases in fluorescence by flow cytometry at 2 min intervals from 0 to 12 min.

^{1}H and ^{13}C attached proton test (APT) experiments (*13*, *14*) were conducted on a Bruker QE Plus 300 NMR spectrometer (Billerica, MA) operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. APT experiments used a spectral width of 20 000 Hz with 64 000 scans and 16 K data points zero filled to 32K data points. APT experiments determine the number of molecular sites that are CH_2 groups (and/or protonless carbon atoms) from those that are CH and CH_3 groups. Fractions collected during HPLC analysis to be used for ^{1}H NMR analysis were first concentrated by evaporation of the solvent mobile phase to about 0.3 mL, 0.5 mL of D_2O was added, it was again evaporated to 0.3 mL, and then made to 0.8 mL with D_2O. The sample was not evaporated to dryness, due to difficulty in resolubilizing the compounds once dry. To detect potential structural peaks, which could otherwise be obscured by a large HDO peak, presaturation (*15*) was used with each of the sample peaks with a spectral width of 3000 Hz with typically 512 scans and 8192 data points per scan. The time required for presaturation using a continuous wave decoupler was varied with the amount of HDO present, typically 1.2 s per scan.

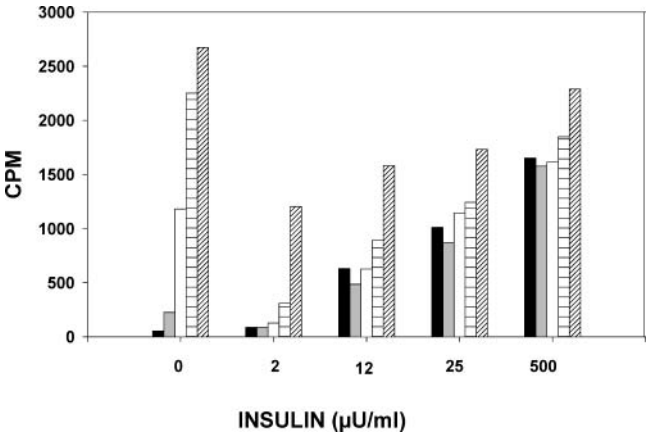

Figure 1. Effects of cinnamon on insulin activity. Solid black bar is the control with no added cinnamon fraction. Second gray bar at each level of insulin denotes purified cinnamon fraction (7 mg/mL) diluted 1:20, open bar denotes 1:10 dilution, horizontal lined bar denotes 1:5, and hatched bar is a dilution of 1:2. 25 μl was added to the 2 mL assay mixture. CPM denotes counts per minute.

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) analyses were performed on an LCQ classic ion trap instrument (ThermoElectron Co., San Jose, CA). Trapped fractions were analyzed by infusion at a flow rate of 5−10 μl/min in the positive ion mode under full scan (*m*/*z* 200−2000 Da) and multiple collision induced dissociations (MS^n) conditions. Typical ESI operating conditions were spray voltage 3.5 kV and capillary at 200 °C. Sheath gas was set at 50% for infusion and 80% for online HPLC. The MS^n collision gas was helium with a collision energy of 24−30% of the 5 V end cap maximum tickling voltage. APCI was operated at 4.5 kV and 400 °C with sheath gas at 80%.

The total ethanol extract was analyzed on an Agilent 1100 HPLC (Wilmington, DE), containing a 150 × 4.6 mm 5 μ Luna C-18 column (Phenomenex, Torrance, CA) operated at 25 °C and initial flow of 0.175 mL/min with a solvent system consisting of acetonitrile/methanol/water (26:14:60, containing 0.1% formic acid).

RESULTS AND DISCUSSION

The insulinlike biological activity of the cinnamon fraction is shown in **Figure 1**. Data are for the insulin-dependent breakdown of radiolabeled glucose to carbon dioxide. Similar results were obtained when glucose incorporation into fat was measured. When no exogenous insulin was added (control), there was still a maximal amount of insulin-dependent activity at the highest level of cinnamon tested (**Figure 1**). Maximal activity was similar at both the highest levels of cinnamon and the highest level of insulin. The activities of the different species of cinnamon tested were not significantly different.

On the basis of the strong insulin-enhancing biological activity of the cinnamon fraction, we measured the bioactivity of a number of compounds derived from cinnamon and other related compounds (**Table 1**). None of the compounds tested displayed any insulinlike or insulin-enhancing activity under our assay conditions.

Table 1. Compounds Found in Cinnamon and Related Compounds Tested[a]

chlorogenic acid	ferulic acid
t-cinnamic acid	guiacol
cinnamic acid methyl ester	homovanillic acid
cinnamide	isovanillic acid
cinnam	2-methoxy-cinnamaldehyde
cinnamyl alcohol	3-methoxy-l-tyrosine
clove oil	4-oxo-4h-1-benzopyan-carboxylic acid
p-coumaric acid	resveratrol
o-coumaric acid	vanillic acid
curcumin	vanillin azine
eugenol	

[a] Compounds were dissolved at 1 mg/mL in 0.1 N ammonium hydroxide or water. If samples were not soluble, dimethysulfoxide was used to improve solubility. 25 μL of samples was assayed directly and at a 10-fold dilution. None of these compounds displayed insulin enhancing activity.

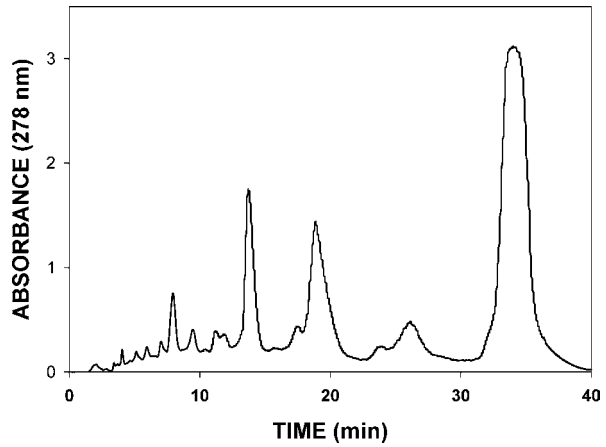

Figure 2. HPLC separation of biologically active cinnamon extract.

It then became critical to isolate and characterize the insulin-potentiating compounds (see Materials and Methods). The major peaks eluting at roughly 14, 19, 26, and 34 min (**Figure 2**) were polyphenol polymers with an absorption maximum at 279 nm. Insulin potentiating activity of these peaks was similar.

Infusion into the MS of the trapped preparative HPLC fractions from the SymmetryPrep C-18 column showed that they consisted of oligomers ranging in detected masses from 576 to 1728 Da. The chromatographic peaks at R_t = 14, 19, 26, and 34 min contained a trimer, tetramer, trimer, and mixture of oligomers, respectively. The trimers and tetramer had molecular masses of 864 and 1152 Da, respectively. The positive ion ESI mass spectra of the trapped fractions showed most of the charge residing on the protonated molecular ion (M + H) with the other ion abundances at less than 25% of the M + H value. Their protonated molecular masses indicated that they were A-type doubly linked procyanidin oligomers of the catechins/epicatechins **(Figure 3)**. In both the ESI and APCI mass spectra, the presence of an ion at *m/z* 287 appears to be indicative of the doubly linked A-type catechin/epicatechin oligomers. Doubly linked type-A catechin/epicatechin oligomers contain C4→C8 carbon and C2→O7 ether bonds between the terminal (T) and middle (M) units of the trimer. Further support for oligomer structure comes from the MS^n experiments conducted on the trimer (M + H, *m/z* 865), in which losses of similar masses from each monomer, in addition to the catechin losses *m/z* 286, 288, and 290 Da, were observed (**Figure 4**). Single linked oligomers of the catechins also display the same characteristic losses (*16*).

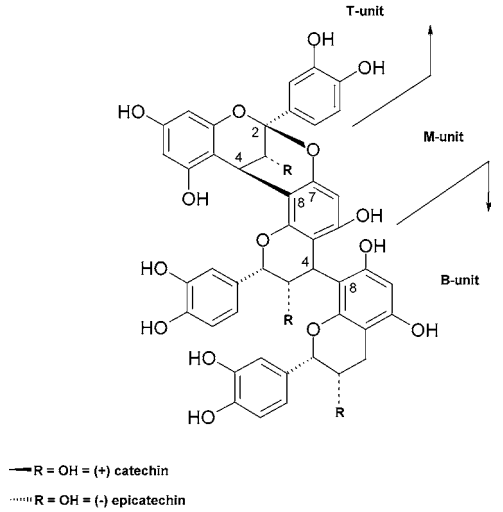

Figure 3. Structure of doubly linked procyanidin type-A polymers found in cinnamon that enhance insulin activity.

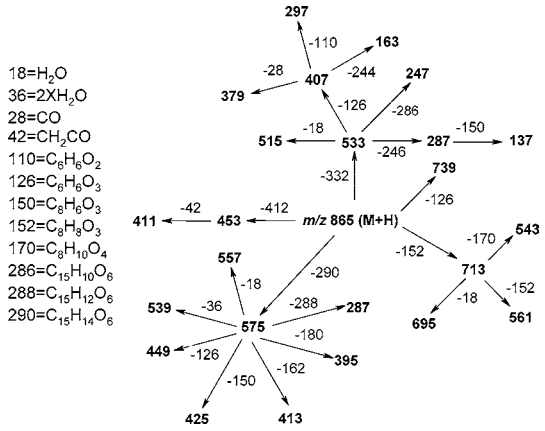

Figure 4. Positive ESI-MS^n dissociation scheme for the doubly linked A-type procyanidin protonated trimer (M + H, *m/z* 865) isolated from cinnamon.

HPLC-APCI-MS ion profiling of the SPE purified ethanol extract for (M + H, *m/z* 865) also showed the presence of minor amounts of additional A-type doubly linked trimers associated with different monomer linkages and/or stereochemistry of the aliphatic hydroxyl groups. Similar ion profiles were observed for other oligomers present in the cinnamon extract. The SPE HPLC-APCI-MS also confirmed the presence of catechin, epicatechin, and procyanidins B1 and B2 based upon their R_t and mass spectra compared to that of authentic material.

An APT experiment was used to differentiate the relative abundance of CH, CH_2, and CH_3 groups. The APT results confirmed that an aromatic region of CH groups and an aliphatic region of CH groups, but only one methylene (CH_2) group, were present. The presence of only one (CH_2) group for a given molecular weight simplified structural elucidation.

The MS result of the peak with a protonated mass of 865 Da fragmented under positive ESI-MS^n conditions into multiple losses of similar masses, consistent with a trimer. Each major fragment on further fragmentation exhibited similar mass losses. The trimer mass formula equals $C_{45}H_{36}O_{18}$, which comprises three $C_{15}H_{12}O_6$ moieties.

The NMR results confirmed the mass spectrometric data. The only CH_2 group is at site 4 in the base unit (B) of the trimer. Each of the three components in the trimer is a close structural analogue of the other. The coupling pattern and chemical shifts

Table 2: Chemical Shift Assignment for Three Trimer Components

proton	T chemical shifts	M chemical shifts	B chemical shifts
H2		3.15 (m)	3.12 (m)
H3	5.56 (s)	4.23 (m)	4.14 (d)
H4	3.53 (d)	3.91 (m)	2.58 (m)
H6	6.13 (d)	6.34 (s)	6.08 (s)
H8	6.30 (d)		
H2′	7.14 (s)	7.14 (s)	7.14 (s)
H5′	6.85 (d)	6.85 (d)	6.98 (d)
H6′	7.36 (s)	7.21 (d)	7.08 (d)

are consistent with the structure as drawn. Eight of the nine sites numbered 2, 3, and 4 are chiral centers. The NMR evidence is that a mixture of stereoisomers are present, and because the stereoisomers have not been separated into enantiomers, assigning the chiral centers as R or S would be beyond the scope of this study. If the three components were exactly equivalent, NMR would not be able to distinguish it from the structure of the monomer.

Each component of the trimer, terminal (T), middle (M) and base unit (B) has minor differences in structure: For the trimer, the M unit would consist of only a single catechin ,and in the case of the tetramer, the middle unit would consist of two catechins. The M and B components with and 8−4 linkage are structurally identical to proanthocyanidin B-1 dimers (*17*). The T, M, and B components have 7, 7, and 8 structurally equivalent protons, respectively (**Table 2**). The NMR spectrum (**Figure 5**) shows that the chemical shift and coupling pattern are consistent with the NMR data for the M and B portion of the trimer. The H2′, H5′, and H6 aromatic peaks are similar in each of the trimers. Consistent with the chemical structure presented, protons for H6 are singlets in M and B; in T both H8 and H6 are present as doublets with a coupling of 3 Hz. The chemical shift values for the aliphatic groups integrate for the nine aliphatic nonexchangeable protons. The aliphatic chemical shifts for the aliphatic protons in T are consistent with a 2,7 as well as 4,8 cross link to M. Two magnetically non equivalent peaks routinely occur for diastereoisomers. Hydrolysis of the 2,7-linkage from T and M during MS analysis would explain why the three monomer components had equal molecular weights. Although the structure is drawn with component M above the plane of the T component, an equally valid structure would be with the M component below the plane of the T component (and the B component below the M component).

Because polyphenols often display antioxidant activity, we determined the activity of the insulin-enhancing fractions (**Figure 6**). There was an inhibition of activity of the production of reactive oxygen species in collagen-stimulated platelets from rats for fractions eluting at 14 and 19 min. The data are means of three separate flow cytometric determinations of fluorescence of the two fractions to reduce the production of the oxidative signals in activated platelets in whole blood samples.

Cinnamon is one of the most frequently consumed spices and is both safe and relatively inexpensive. Furthermore, during aqueous extraction, the overwhelming majority of the lipid-soluble components of cinnamon bark would remain in the insoluble fraction. In the NMR spectrum of the partially purified extract, much less than 10% of the total organic material in solution was identified as cinncassiols (hydroxyl-substituted diterpenoids), saturated hydrocarbons, or other unidentified compounds (data not shown). The lipid soluble fraction of cinnamon contains the phytochemicals most likely to be toxic at higher doses, or with chronic cinnamon ingestion (*8*, *15*, *18*). The cinnamon “essential oil”, containing mainly terpenes,

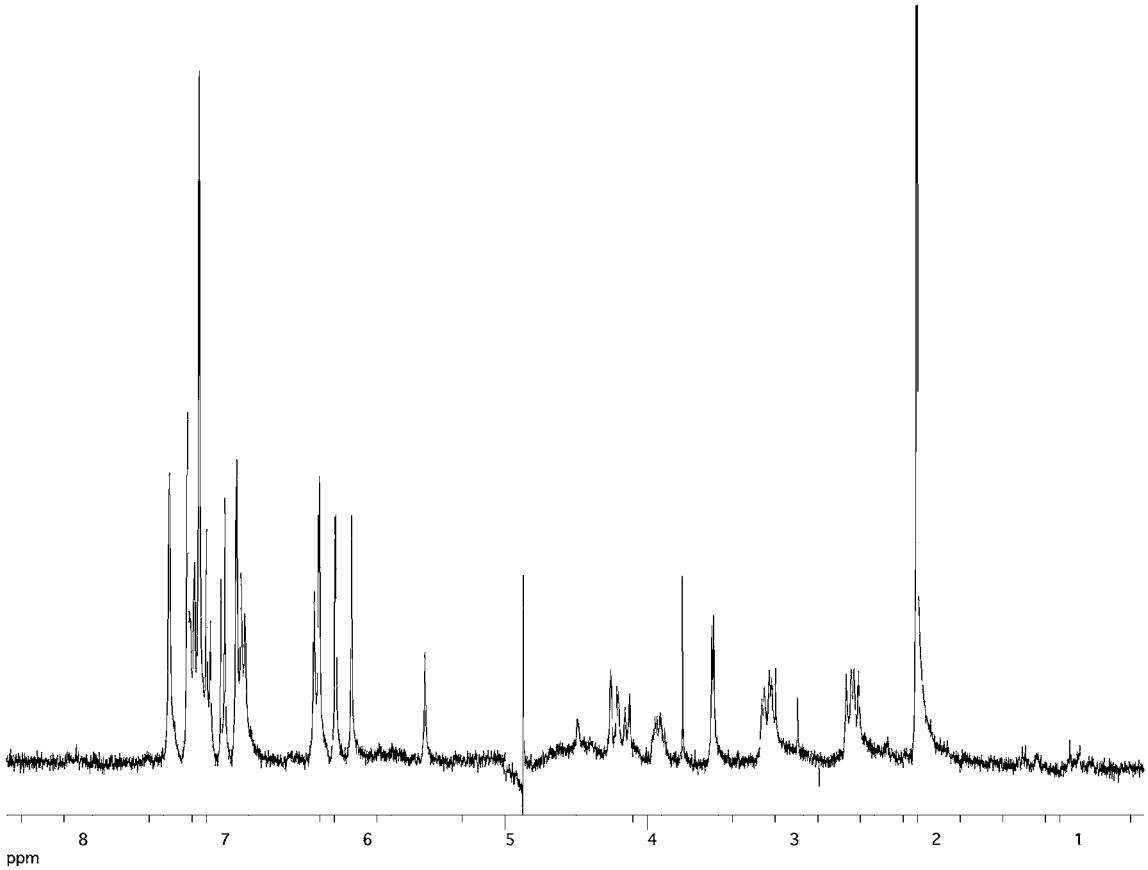

Figure 5. NMR spectrum of purified water soluble cinnamon fraction.

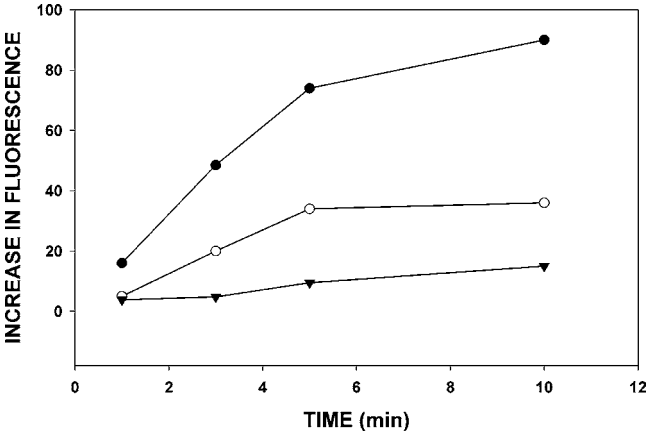

Figure 6. Inhibition of production of reactive oxygen species in platelets by cinnamon fractions. (●) Denotes control; (○) fraction eluting at 14 min, and (▼) fraction eluting at 19 min. (see **Figure 2**).

aldehydes, and eugenol is not present or present at very low levels in the water soluble insulin potentiating fractions; therefore, these fractions are not likely to have significant toxicity at physiological doses. The insulin-enhancing biological activity of the polymers isolated from cinnamon is rather unique as none of the cinnamon or cinnamonlike compounds we tested displayed any biological activity in the insulin potentiation assay (**Table 1**).

The polyphenolic polymers have antioxidant effects, which may provide synergistic benefits for the treatment of diabetes. Botanical antioxidants may play a role in helping maintain the integrity of cell membranes by preventing polyunsaturated fatty acid peroxidation. The lipid composition of muscle membrane phospholipids reflects the combined influences of diet and desaturase/elongase activity, and in turn, the phospholipid composition affects the binding and action of insulin. In general, the more unsaturated the membrane, the better glucose is utilized. The more saturated the membrane, the more deleterious the effects on insulin efficiency (*19*).

Lipid peroxidation and damage by reactive oxygen metabolites are also major problems in terms of diabetic complications. Clinical studies have shown that supplementation of both nutrient and phytochemical antioxidants can reduce or slow the progression of various complications of diabetes (20−*22*).

Catechin procyanidins have been reported in a number of products (*23*−*25*). Most of the procyanidins reported are the single linked B-type procyanidins. We report here that the major procyanidin oligomers in the ethanolic and aqueous extracts of cinnamon are doubly linked type-A. These polymers are consistent with the oligomeric procyanidins reported to be present in aqueous fractions from *Cinnamonium cassia* (*24*) and are likely doubly linked A-type mers (*25*) composed of repeating mass units of 288.

These studies demonstrate that water-soluble polymeric compounds isolated from cinnamon have insulin-enhancing biological activity in the in vitro assay measuring the insulin-dependent effects on glucose metabolism and also function as antioxidants. These same compounds have been shown to inhibit phosphotyrosine phosphatase in the insulin-receptor domain and to activate insulin receptor kinase (*26*) and function as a mimetic for insulin in 3T3-L1 adipocytes (*27*).

These results suggest that compounds present in cinnamon may have beneficial effects on glucose, insulin and blood lipids and may prove to be beneficial in the treatment of diabetes. More than 100 million people worldwide die each year from diabetes, and for many, drugs or other forms of treatment are unavailable. It may be possible that many of these people could benefit from readily available natural products such as cinnamon that could have profound effects on their overall health. During the completion of this work, we completed a human study involving subjects with type 2 diabetes consuming cinnamon (*28*). Subjects consumed 1, 3, or 6 g of cinnamon per day for 40 days with 3 placebo groups corresponding to the three groups that consumed different numbers of capsules containing cinnamon. There were significant decreases in fasting serum glucose (18−29%), triglycerides (23−30%), total cholesterol (12−26%), and LDL cholesterol (7−27%) after 40 days. Values after 20 days were intermediate. After 40 days, subjects stopped taking cinnamon from days 40−60. Values after 60 days were returning to prestudy levels but were largely lower than initial values. There were no significant changes in the three placebo groups. The responses to 1, 3, and 6 g of cinnamon per day were similar, suggesting that even lower levels of cinnamon may elicit improvements. The magnitude of these effects due to taking a common spice illustrate the importance of naturally occurring insulin-enhancing complexes in the prevention and alleviation of glucose intolerance and diabetes. Benefits in insulin sensitivity are also likely to lead to decreased incidence of cardiovascular diseases, which are more than double in people with diabetes.

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