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Additive or synergetic effects of phenolic compounds on human low density lipoprotein oxidation

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Abstract

The in vitro assessment of the antioxidant capacity of four phenolic compounds; catechin, hesperidin, ferulic acid, and quercetin was evaluated by the examination of their ability to inhibit copper (Cu^{2+})-mediated human low density lipoprotein (LDL) oxidation by using the thiobarbituric acid-reactive substances (TBARS) assay. Individually, the enrichment of LDLs with various concentrations of catechin, hesperidin, ferulic acid, and quercetin produced both antioxidant and prooxidant effects depending on enrichment concentrations of the polyphenolic compounds. Catechin and hesperidin had predominantly antioxidant effects (51.1%, 76.9%, respectively) while ferulic acid and quercetin had mostly prooxidant effects (166.4%; 191.8%, respectively) on LDL oxidation. However, when the mixture of the four phenolic compounds was used to enrich the LDLs, significant antioxidant capacity was demonstrated at all enrichment levels with a dose–response. Synergistic effects of the polyphenolic compounds as mixtures in preventing human LDL oxidation may reflect that nutritional advantages are found in the consumption of a variety of fruits and vegetables in preventing LDL oxidation and perhaps a host of cardiovascular diseases.
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1. Introduction

Phenolic compounds, also known as polyphenols and bioflavonoids comprise one of the largest and most ubiquitous groups of plant metabolites with more than 4000 structures already identified (Middleton et al., 2000; Ross and Kasum, 2002; Dubick and Omaye, 2001; Cheynier, 2005). These substances possess an aromatic ring bearing one or more hydroxyl substituents (Morton et al., 2000). All plant phenolic compounds arise from the common intermediate, phenylalanine, or its close precursor, shikimic acid (Yang et al., 2001) through two main synthetic pathways: the acetate pathway and the shikimate (polyketide) pathway, respectively (Ross and Kasum, 2002). Such compounds, initially studied for its function as plant pigmentation (Middleton et al., 2000) and flavoring (Ross and Kasum, 2002) are formed to protect the plant from photosynthetic and environmental stress (Grace and Logan, 2000), reactive oxygen species (Harborne and Williams, 2000; Ross and Kasum, 2002), wounds and herbivores (Bravo, 1998).

Recent research has also shown interest in phenolic compounds antioxidative, antiinflammatory, and anticarcinogenic activities. Epidemiological and experimental studies in animals and humans suggest that flavonoids may reduce the risk of cardiovascular diseases (CVD) (Geleijnse et al., 1999; Hertog et al., 1993; Keli et al., 1996; Liu et al., 2000; Sesso et al., 2003), cerebrovascular diseases (Knekt et al., 2002; Truelsen et al., 1998), and cancers (Arts et al., 2001). A balanced diet abundant in foods of plant sources can significantly slow down the development of CHD (Grundy, 1986; American Heart Association, 1988; Feldman, 1994).

Abbreviations: LDL, Low density lipoproteins; TBARS, Thiobarbituric acid-reactive substances.

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Oxidation of polyunsaturated lipid components of low density lipoproteins (LDL) by active oxygen species may explain apparent abnormalities observed in attempts to relate heart disease exclusively to fat intakes. It is believed that the oxidation of LDL plays a key role in the pathogenesis of atherosclerosis (Esterbauer, 1993; Esterbauer et al., 1992; Steinberg et al., 1989; Witztum and Steinberg, 1991). Oxidation reactions are propagated by peroxyl radicals and, therefore, the capacity of an individual LDL particle to scavenge these oxidants may be an important indicator of its atherosclerotic potential. Although much is known about the role of each antioxidant (at certain levels of intake) on LDL oxidation, there is a limited amount of data on intakes of antioxidant mixtures (Ascherio and Willett, 1995; Omaye, 1998; Omaye and Zhang, 1998). Likewise, our understanding of intakes of bioactive compound mixtures, such as various polyphenolics, which provide optimal protection against oxidized LDL, is limited.

The present study was designed to determine the efficacy of four phenolic compounds (ferulic acid, quercetin, catechin, and hesperidin) in the protection against LDL oxidation. The four compounds were chosen for this study based on their prevalence in a wide range of food sources, thus realistically mimic what is found in the human diet. Ferulic acid is linked through ester bonds to hemicellulose of the cell wall and thus found in food sources, such as wheat bran (5 mg/g). Two ferulic acid molecules linked by a methylene, with a β-diketone structure in a highly conjugated system, form curcumin, the major yellow pigment in turmeric and mustard. It is used widely as a food preservative and yellow coloring agent for foods, drugs, and cosmetics (Yang et al., 2001). Quercetin is the main flavonol (a subclass of the flavonoids, the largest class of phenolic compounds) in our diet and is present in many fruits and vegetables as well as in beverages. It is particularly abundant in onions (0.3 mg/g fresh weight) and tea (10–25 mg/L) (Hertog et al., 1993). Catechin is also a flavonol and is found primarily in tea, red wine, and chocolate (Yang et al., 2001). Citrus fruits are the main food sources of flavanones (also a subclass of the flavonoids) and the most widely consumed flavonone is hesperidin from oranges (125–250 mg/L of juice) (Scalbert and Williamson, 2000).

2. Materials and methods

2.1. Human subjects

Sixty-five healthy students, between 20 and 35 years of age, participated in this study. Individuals who indicated any of the diseases previously described were dismissed from this project. The present study was approved by the University of Nevada, Reno Biomedical Institutional Review Board. Participants were asked to sign the approved informed consent forms. Blood samples (40 mL) were collected by venipuncture from fasted individuals, in subdued light, and immediately placed on ice in the dark. Blood samples were centrifuged (Sorvall RT6000B Refrigerated Centrifuge, DuPont Corporation, Wilmington, Delaware) at 1500*g* (6000 rpm) 4 °C for 15 min to separate the plasma fraction. To exclude individuality effects, plasma from all subjects was pooled prior to enrichment with the phenolic compounds.

2.2. Enrichment with phenolic compounds

The compounds used alone or within mixtures were catechin ((+)-*trans*-3,3′,4′,5,7-pentahydroxyflavane), hesperidin (Hesperetin 7-rhamnoglucoside), ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid), and quercetin (3,3′,4′,5,7-pentahydroxyflavone dehydrate) (Sigma–Aldrich, St Louis, MO). Five concentrations (20, 9.3, 4.3, 2, 0 μMol/L) of each phenolic compound tested were added to plasma. The concentrations used were based on literature cited as to the typical phenolic concentrations used for observation of oxidation in blood or plasma (hesperidin (El-Shafae and El-Domiaty, 2001); catechin and quercetin (O'Reilly et al., 2000); quercetin: (Miranda et al., 2000); catechin (Andrikopoulos et al., 2002; Kim et al., 2000; Leung et al., 2001; Lotito and Fraga, 2000). A mixture of all four phenolic compounds used in this experiment was also tested in the prevention of LDL oxidation. The concentrations for the mixture were determined by adding the original volume of each phenolic compound at the concentration used for individual analysis (e.g., at concentration 20 mMol/mL, 50 μL of individual phenolic compounds was added to plasma at a total volume of 200 μMol/L). Tubes with enriched plasma were stored under nitrogen and subsequently incubated in a 37 °C water bath (Fisher Waterbath, Fisher Scientific, Fair Lawn, New Jersey) in the dark for 6 h.

2.3. LDL isolation

Low-density lipoprotein (LDL) was isolated according to the single vertical spin density gradient ultracentrifugation method described by Chung et al. (1986). LDL was separated by single vertical spin density gradient ultracentrifugation at 4 °C, 63,000 rpm for 3.75 h (3 h, 45 min) in a Beckman L2-85 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA), using a fixed-angle Beckman 70.1 Ti rotor (Beckman Instruments, Inc., Fullerton, CA). The LDL fraction was collected by puncturing the tube with the needle of a syringe and collecting the yellow/orange layer that formed in the top third of the tube. The LDL tubes were stored under nitrogen at −70 °C. The LDL was then filtered with 25 mL of PBS using Amersham Pharmacia Columns PD-10 containing Sephadex® G-25 (Amersham Pharmacia Biotech, Wikstroms, Sweden).

2.4. Measurement of protein concentration

Protein concentration was determined following the Warburg–Christian method (Alexander, 1993). Protein concentrations were first estimated by direct absorption measurement in the ultraviolet (UV) region in a spectrophotometer (UV/VIS/NIV Spectroscopy, Perkin–Elmer, Norwalk, CT). Following the measurement of the absorption maximum values of protein, the determination of the optical density of the LDL solution at 280 nm was determined by using a normograph (Calbiochem, San Diego, CA).

2.5. Thiobarbituric acid-reactive substances

Oxidation of LDL was measured by the thiobarbituric acid-reactive substances (TBARS) assay of Buege and Aust (1978). LDL was oxidized in vitro, by incubation with Cu^{2+} ions (50 μM $CuSO_4$). Samples were incubated in sealed separate tubes to preserve the volume and were removed as needed. The length of the lag period appears to reflect the natural lag period of autocatalytic lipid peroxidation (Halliwell and Gutteridge, 1998). The time course begins with the combination of a 180 μL LDL sample with 360 μL of a mixture containing 15% trichloroacetic acid (w/v): 0.375% thiobarbituric acid (w/v): 0.25 N hydrochloric acid (TCA–TBA–HCl) and 1 μL of $CuSO_4$ (50 μM). Samples were placed in a 100 °C water bath for 15 min and cooled on ice. The procedure was identically repeated at 10, 20, 30, 45, 60, 120, 240, 360, 480, and 600 min.

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