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Chemical characterization and chemo-protective activity of cranberry phenolic powders in a model cell culture. Response of the antioxidant defenses and regulation of signaling pathways

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ABSTRACT

Oxidative stress and reactive oxygen species (ROS)-mediated cell damage are implicated in various chronic pathologies. Emerging studies show that polyphenols may act by increasing endogenous antioxidant defense potential. Cranberry has one of the highest polyphenol content among commonly consumed fruits. In this study, the hepato-protective activity of a cranberry juice (CJ) and cranberry extract (CE) powders against oxidative stress was screened using HepG2 cells, looking at ROS production, intracellular non-enzymatic and enzymatic antioxidant defenses by reduced glutathione concentration (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR) activity and lipid peroxidation biomarker malondialdehyde (MDA). Involvement of major protein kinase signaling pathways was also evaluated. Both powders in basal conditions did not affect cell viability but decreased ROS production and increased GPx activity, conditions that may place the cells in favorable conditions against oxidative stress. Powder pre-treatment of HepG2 cells for 20 h significantly reduced cell damage induced by 400 μM *tert*-butylhydroperoxide (t-BOOH) for 2 h. Both powders (5–50 μg/ml) reduced *t*-BOOH-induced increase of MDA by 20% (CJ) and 25% (CE), and significantly reduced over-activated GPx and GR. CE, with a significantly higher amount of polyphenols than CJ, prevented a reduction in GSH and significantly reduced ROS production. CJ reversed the *t*-BOOH-induced increase in phospho-c-Jun N-terminal kinase. This study demonstrates that cranberry polyphenols may help protect liver cells against oxidative insult by modulating GSH concentration, ROS and MDA generation, antioxidant enzyme activity and cell signaling pathways.

1. Introduction

Oxidative stress and reactive oxygen species (ROS)-mediated cell damage have been implicated in the development of various human chronic pathologies such as cardiovascular disease, certain cancers and a number of neurodegenerative diseases (Sohal, Mockett, & Orr, 2002). Epidemiological studies have related a diet rich in fruits and vegetables to the prevention of chronic degenerative diseases linked to oxidative stress (Cassidy, Huang, Rice, Rimm, & Tworoger, 2014; Ramos, 2008; Wedick et al., 2012).

Fruits, including berries, are one of the most important food sources of phenolic compounds in our diets (Caillet, Côté, Doyon, Sylvain, & Lacroix, 2011). The North American cranberry (*Vaccinium macrocarpon* Ait. Ericaceae) is of growing public interest as a functional food because of potential health benefits linked to the elevated content of bioactive phytochemicals in the fruit. In fact, cranberry ranks high among fruits in both antioxidant quality and quantity (Caillet et al., 2011; He & Liu, 2006; Vinson, Su, Zubik, & Bose, 2001) because of its substantial flavonoid and phenolic acid content. Cranberries, in powder, extract or juice, have been reported to be beneficial for the treatment of urinary tract infections (Blumberg et al., 2013; Pérez-López et al. 2009; Wang et al., 2012), obesity, insulin resistance and intestinal inflammation (Anhe et al., 2014), management of hyperglycemia (Da Silva Pinto, Ghaedian, Rahul Shinde, & Shetty, 2010; Kim, Kim, & Kwak, 2014), diabetes and hypertension (Apostolidis, Kwon, & Shetty, 2006), and hypercholesterolemia (Kim et al., 2014) and have shown antiproliferative activity in cancer cells (He & Liu, 2006; Seeram et al., 2006).

These and other health benefits, including reduced risks of cancer and cardiovascular disease, are believed to be due to the presence of various polyphenolic compounds, including flavonoids such as flavonols, flavanols (monomeric and polymeric) and anthocyanins, and phenolic acids such as coumaric and chlorogenic, as well as the synergistic effects among them (Chu & Liu, 2005; Heinonen, 2007; Seeram, Adams, Hardy, & Heber, 2004). In vitro evidence has shown the antioxidant potential of cranberry phenolic compounds (Borges, Degeneve, Mullen, & Crozier, 2010) while the antioxidant and chemo-protective

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properties of individual food flavonoids or polyphenolic extracts have been widely reported in cultured cells (Ramos, 2008), animal models (Mukhatar & Ahmad, 2000) and humans (Ahn et al., 2003; Bettuzzi, Rizzi, & Belloni, 2007). A recent publication also showed the bioavailability and bioactivity of cranberry phenolics from consuming cranberry juice (McKay, Chen, Zampariello, & Blumberg, 2015).

The protective effect of these compounds is related to their function in sequestering ROS and/or maintaining the cell components in their correct redox state, but emerging findings indicate that natural compounds may also act by increasing the endogenous antioxidant defense potential through regulation of cell signaling pathways (Martín et al., 2010a; Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005; Ramos, 2008). It is known that mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinase (PI3K/AKT) are enhanced in response to treatments with dietary compounds (Chen & Kong, 2004; Ramos, 2008). MAPKs family includes members such as extracellular-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK) and p38. These MAPKs and PI3K/AKT pathways are transducers of a multitude of extracellular stimuli by phosphorylating and activating downstream transcription factors enabling the cell to respond to stress by increasing or decreasing the expression of critical genes (Chen & Kong, 2004; Ramos, 2008). Several studies have shown that chlorogenic acid (Granado-Serrano et al., 2007), epicatechin (Granado-Serrano et al., 2007; Granado-Serrano et al., 2010) and quercetin (Granado-Serrano, Martín, Bravo, Goya, & Ramos, 2012) induce a varied set of antioxidant mechanisms in diverse organs or cultured cells through the regulation of protein kinases involved in cell signaling pathways.

The liver is particularly susceptible to toxic and oxidative abuse since the portal vein brings blood to this organ after intestinal absorption. The absorbed drugs and xenobiotics in a concentrated form can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic stress (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006). Therefore, studies dealing with the effect of antioxidants at a physiological level in liver of live animals and at a cellular level in cultured hepatic cells are necessary. Human liver HepG2 is widely used for biochemical and nutritional studies as a cell culture model in human hepatocytes since they retain their morphology and most of their function in culture (Brandon et al., 2006; Goya, Martín, Ramos, Mateos, & Bravo, 2009). Previous cell culture studies have demonstrated that several flavonoids (Kanazawa, Uehara, Yanahitani, & Hashimoto, 2006) and hydroxycinnamic acids (Mateos, Goya, & Bravo, 2006) are absorbed and metabolized by HepG2 cells. These cells are able to express main antioxidant defense enzymes such as catalase, glutathione peroxidase (GPx), glutathione reductase (GR) on par with or better than that of primary human hepatocytes (Lee et al., 2002; Mersch-Sundermann, Knasmuller, Wu, Darroudi, & Kassie, 2004). Thus, in this study, HepG2 cells were utilized to screen for hepato-protective activity of two cranberry powders against an oxidative challenge induced by the potent oxidant *tert*-butyl hydroperoxide (*t*-BOOH). In addition, the involvement of PI3K/AKT and MAPK proteins in the protective response to oxidative stress in these cells was also investigated.

2. Methods and materials

2.1. Reagents

Tert-butylhydroperoxide (*t*-BOOH), o-phthalaldehyde (OPT), glutathione reductase (GR), reduced (GSH) and oxidized glutathione, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,4-dinitrophenylhydrazone (DNPH), gentamycin, penicillin G, streptomycin, β-mercaptoethanol and EDTA were purchased from Sigma-Aldrich (Madrid, Spain). Benzoic acid, salicylic acid, protocatechuic acid, gallic acid, vanillic acid, *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, ellagic acid, quinic acid, galacturonic acid, catechin, epicatechin, quercetin, 4-dimethylaminocinnamaldehyde (DMAC) and Sephadex LH-20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercitrin, hyperoside, myricetin, myricetrin, procyanidin A2, cyaniding-3-galactoside and peonidin-3-glucoside were purchased from Indofine Chemical Company, Inc. (Hillsborough Township, NJ, USA). Cyanidin-3-glucoside was purchased from ChromaDex (Irvine, CA, USA). Citric acid, malic acid, glucose and fructose were purchased from Fisher Scientific (Waltham, MA, USA). Anti-AKT and antiphospho-Ser473-AKT (p-AKT), anti-ERK1/2 and antiphospho-ERK1/2 (p-ERKs) recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK1/2, and antiphospho-JNK1/2 (p-JNKs) recognizing phosphorylated Thr183/Tyr185 of JNK1/2 and anti-ß-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Bradford reagent was from BioRad Laboratories (Madrid, Spain). All reagents were of analytical or chromatographic quality.

2.2. Cranberry powder preparation

Cranberry extracts were obtained from Ocean Spray Cranberries (MA, USA). Cranberry juice (CJ) powder was prepared from the juice of mature berries of the commonly cultivated cranberry plant (*Vaccinium macrocarpon*). Cranberry juice processing consists of the milling and pressing of the berries after a hot (50 °C for 1 h) commercial pectinase maceration. CJ was then prepared by spray drying cranberry concentrate with magnesium hydroxide as the carrier and tri-calcium phosphate as an anti-caking agent. The powder is fine, free-flowing and rosy red in color, and contains approximately 90% cranberry solids. The cranberry extract (CE) powder is a water-soluble, phenolic-rich extract of cranberry utilizing a proprietary resin separation process. CE was standardized to 55% proanthocyanidin (PAC) content on a dry weight basis as analyzed by the 4-dimethylaminocinnamaldehyde (DMAC) method. Finally, 10 mg/mL stock solutions of CJ and CE were prepared in distilled water for cell treatment.

2.3. Chemical characterization of cranberry powders

2.3.1. HPLC-MS/MS analysis of individual phenolics, flavonols and flavanols

Individual phenolic acids, flavonols and flavanols in the cranberry extracts were analyzed by HPLC-MS/MS (Borges et al., 2010) after the powders were dissolved in water (400 mg cranberry juice powder in 25 mL distilled water and 100 mg cranberry extract powder in 25 mL of distilled water). HPLC analyses were made with an Agilent 1260 infinity series LC system (Agilent technologies, Palo Alto, CA, USA) equipped with a binary pump, autosampler and coupled with a QTRAP 4000 mass spectrometer (AB Sciex, Framingham, MA, USA). Separation was carried out on a Phenomenex Kinetex 2.6 u C18, 100 Å 150 × 3.0 mm column (Torrance, CA, USA). Gradient elution was conducted, using: 0.1% formic acid/deionized water (phase A) and 0.1% formic acid/acetonitrile (phase B) at a constant flow of 0.5 mL/min using the following gradient: 0 – 4 min, isocratic of 5% B; 4 – 26 min, linear gradient from 5% B to 45% B; 26 – 30 min linear gradient from 45% B to 100% B; and 30 – 35 min, isocratic of 100% B. The injection volume was 15 μL and column temperature set at 40 °C. All the analyses were performed using the Turbo V ionization source in negative ion mode with the following settings: curtain gas (CUR) 25 (arbitrary units), collision gas (CAD) high, ion spray voltage (IS) −4000 V, ion source gas 1 (GS1) and 2 (GS2) 60 (arbitrary units). The drying gas (N2) was heated to 600 °C. The declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized for all the compounds using standard material (Table 1). Compounds were identified by comparing their retention time, MS and MS/MS fragmentation spectra with those obtained from pure standard solutions. Compounds were quantified by multi reaction monitor (MRM) experiments, considering one MS/MS transition for each compound.