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Effect of pecan phenolics on the release of nitric oxide from murine RAW 264.7 macrophage cells

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ABSTRACT

Inflammation is linked to numerous chronic disease states. Phenolic compounds have attracted attention because a number of these compounds possess anti-inflammatory properties. A phenolic crude extract was prepared from pecans and separated by Sephadex LH-20 column chromatography into low- and high-molecular-weight (LMW/HMW) fractions. Anti-inflammatory properties of these fractions were assessed in LPS-stimulated RAW 264.7 murine macrophage cells. NO and reactive oxygen species (ROS) production was monitored after 3 different experimental protocols: (1) pre-treatment with *Escherichia coli* O111:B4 lipopolysaccharide (LPS); (2) pre-treatment with a pecan crude extract and its fractions; and (3) co-incubation of LPS with a pecan crude extract and its fractions. The LMW fraction displayed a dose-dependent decrease in NO production and a significant decrease from the LPS control in ROS production when cells were either co-incubated with or pre-treated with LPS. The phenolics were characterized by HPLC to help identify those responsible for the observed effect.

1. Introduction

NF-κB is a protein complex shown to play a key role in the inflammation process in macrophages and lymphocytes (Glass & Witztum, 2001). During normal physiological conditions, the protein complex is located in the cytosol of the cell as an inactive complex, IκB-NFκB (Baeuerle & Baltimore, 1996). The inflammatory process begins when one of several possible stimuli activates the cell. These stimuli can be exogenous (such as lipopolysaccharides, LPS) or endogenous (TFN-α or IFN-γ) (Rajapakse, Kim, Mendis, & Kim, 2008; Terra et al., 2007). The stimulation then causes the enzyme IκB-kinase (IκK) to phosphorylate the inactive complex. Once the complex is activated, it can pass into the nucleus where it can stimulate gene expression for the inducible nitric oxide synthase (*i*NOS) (Aktan, 2004; Kretz-Remy, Mehlen, Mirault, & Arrigo, 1996; Rajapakse et al., 2008), and a marked increase in the production of cytokines and other inflammatory mediators (Barnes & Karin, 1997). Nitric oxide (NO), a somewhat-stable free radical, is thought to be a mediator of inflammation (Skoog et al., 2002) and is produced once the NF-κB complex has been activated by extracellular stimuli (Kumar, Abbas, Fausto, & Aster, 2010; Terra et al., 2007). Moreover, it is generated during everyday activities to help mediate other biological functions in the human body (Skoog et al., 2002). The conditionally-indispensible amino acid L-arginine is converted to NO by one of three NO synthases: constitutive synthases, endothelial (*e*NOS) and neuronal nitric oxide synthases (*n*NOS), as well as inducible nitric oxide synthase (*i*NOS). Different from the constitutive synthases, *i*NOS is not found in uninjured cells (Alderton, Cooper, & Knowles, 2001; Mayer & Hemmens, 1997). Upon any form of injury or inflammation, the NF-κB complex will, however, initiate cytokines in the cell to begin producing *i*NOS (Qidwai & Jamal, 2010).

NO serves multiple purposes in the inflammation process (Laroux et al., 2001). It is a small molecule and is therefore capable of diffusing easily into cells. This enables the NO to travel from the site of formation to various sites of action (Aktan, 2004; Kruidenier & Verspaget, 2002). Because it is a free radical, NO has the potential to react with other free radicals found in cells. NO itself is not extremely reactive, but it does form a much more damaging reactive nitrogen species (RNS), peroxynitrite anion ($ONOO^-$) (Zhong, Chiou, Pan, & Shahidi, 2012). For this reason, the pro-oxidative NO has been shown to create oxidative damage (Epe, Ballmaier, Roussyn, Briviba, & Sies, 1996; Luperchio, Tamir, & Tannenbaum, 1996; Virgili, Kobuchi, & Packer, 1998). It is also believed that ROS may play a key role in the activation of NF-κB (Brigelius-Flohé, Banning, Kny, & Böl, 2004).

Studies have investigated the anti-inflammatory properties of pine bark extract (Virgili et al., 1998), cocoa procyanidins

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(Zhang et al., 2006) and a hydrophilic phenolic extract from pistachios (Gentile et al., 2012) in cell culture systems. Varying, and conflicting, results have been reported in regards to which extract constituents are most effective at reducing NO and ROS production. For instance, one study reports that HMW tannin constituents (Gentile et al., 2012) are the most effective, while others have demonstrated the effectiveness of monomers and dimers (Park, Rimbach, Saliou, Valacchi, & Packer, 2000; Zhang et al., 2006). Due to the moderately high proanthocyanidin (PAC) content of pecans, it was postulated that a phenolic crude extract from pecans would be excellent as inhibitors of the inflammatory process. The prepared pecan crude extract was further separated via Sephadex LH-20 column chromatography to give two phenolic fractions: a LMW and a HMW one. Through the combination of results from HPLC characterization and cell culture assays, our objective is to indicate which compounds may be most effective as anti-inflammation agents.

2. Materials and methods

2.1. Chemicals and glassware

Sea sand, glass wool, dimethyl sulfoxide (DMSO), sodium carbonate, and ethanol (95%), as well as ACS-grade methanol, hexanes, and acetone plus HPLC-grade water, methanol and acetonitrile were acquired from Fisher Scientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid was purchased from VWR International, LLC (Suwanee, GA, USA). *Escherichia coli* O111:B4 lipopolysaccharide (LPS), Triton X-100, fetal bovine serum (FBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Folin & Ciocalteu's phenol reagent and (+)-catechin hydrate were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM/high glucose + sodium pyruvate), L-glutamine, penicillin-streptomycin, and phosphate-buffered saline (PBS) were ordered from Invitrogen, Inc. (Grand Island, NY).

2.2. Sample collection

Pecans (Desirable variety, random selection from five orchards in South Georgia) were harvested during the autumn of 2012 and shipped to the Department of Food Science & Technology, Athens, GA. The nuts were raw and remained in-shell through transport, packaging, and storage. After arrival in Athens, the samples were placed in labelled pouches (Sealed Air Corporation, Elmwood Park, NJ), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to help prevent oxidative degradation, and stored at −80 °C until analyzed. On the day of analysis, pecan samples were removed from the freezer, cracked, shelled, and tempered to room temperature to ensure a proper mass reading. Approximately 15 g of nutmeat were placed into a −20 °C freezer and allowed to partially refreeze to facilitate grinding. Each nut sample was then combined with ~65 g of washed sea sand and ground in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ), to a very fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nutmeat during the particle size reduction process.

2.3. Extraction of phenolic compounds

A Soxhlet apparatus was employed to remove lipids from all pecan samples. The $(CH_3)_2CO/H_2O/CH_3COOH$ solvent mixture (70:29.5:0.5 v/v/v, 100 ml) of Wu et al. (2004) was used to extract phenolic compounds from the defatted nutmeat. Extraction was carried out according to Craft, Kosińska, Amarowicz, and Pegg (2010). Briefly, the contents in the flasks were heated at 50 °C for 30 min in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ). The extraction was performed in triplicate, and supernatants were pooled. Acetone was evaporated from the supernatant using a Rotavapor. The aqueous portion was transferred to crystallization dishes, covered with filter paper, and placed in a −80 °C freezer until frozen. The samples were then lyophilized (Labconco Freezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MS). The dried extract was placed in amber-coloured vials, capped, and stored at 4 °C until ready for use. The entire extraction process was conducted in triplicate.

2.4. Fractionation of the phenolic extracts

Pecan phenolics were fractionated from the crude extracts according to Strumeyer and Malin (1975) with slight modifications. Briefly, a ~2 g sample of pooled pecan crude extracts from 5 independent preparations was mixed in a small volume of ~75% (v/v) ethanol and sonicated to ensure that it was completely dissolved. Using a Pasteur pipette, the sample was applied to the top of a chromatographic column packed with Sephadex LH-20 (bead size: 25–100 μm; Chromaflex column, 30 × 400 mm [I.D. × length], Kontes, Vineland, NJ, USA). LMW phenolic compounds were eluted with ~1.5 L of 95% (v/v) ethanol. Fractions (15 ml) were collected using a Beckman Coulter SC100 fraction collector (Fullerton, CA, USA). The eluent was then switched to 50% (v/v) aqueous acetone and a HMW phenolic fraction was eluted from the column with ~600 ml of this mobile phase.

2.5. Pooling of isolated fractions

Each 15 ml LMW phenolic fraction was analyzed using an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) by scanning across a wavelength range of 200–400 nm. Spectral differences at key wavelengths (i.e., λ = 255 nm {ellagic acid and its derivatives}, 278 nm {phenolic acids, catechin, epicatechin}, 320 nm {phenolic acids of the *trans*-cinnamic acid family} and ~360 nm {flavonols}) were used as a guide to group the collected fractions into 5 pooled lots (Fractions I–V); pooling was based on observed similarities and differences in the spectra of the samples. Further details of the pooling process as well as a depiction of the spectral characteristics for the resultant fractions are given by Robbins, Ma, Wells, Greenspan, and Pegg (2014). After pooling, ethanol was evaporated using a Rotavapor with the water bath set at 45 °C. To ensure complete removal of solvent and moisture, the pooled fractions were lyophilized with the Labconco freeze dryer and then stored in amber-glass bottles at 4 °C until analysis. Apropos the HMW fraction: acetone was also removed *in vacuo* and the aqueous residue lyophilized and stored as described above.

2.6. Total phenolics content (TPC) determination

The TPC assay of the ethanolic fractions was determined based on a method adapted from Swain and Hillis (1959), employing Folin & Ciocalteu's phenol reagent. Each fraction was diluted to 0.20 mg/ml in anhydrous methanol. The assay was performed using 1 ml of methanolic extract, 7.5 ml deionized water, 0.5 ml Folin & Ciocalteu's phenol reagent, and 1 ml of saturated sodium carbonate. The resultant solution was vortexed for 30 s. The tubes sat for 60 min to allow for maximal colour development. The absorbance of the resulting chromophore from this colorimetric reaction was measured at λ = 750 nm using the Agilent spectrophotometer. A standard curve was prepared using known concentrations of working solutions of (+)-catechin ranging from 1.6 to 8.0 μg/ml.