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Anti-inflammatory effects of characterized orange peel extracts enriched with bioactive polymethoxyflavones

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Abstract

In view of the potential of polymethoxyflavones (PMFs) and hydroxylated PMFs (OH-PMFs) as bioactives against inflammation, we prepared six different orange peel extracts (OPEs). The major compounds of these extracts were characterized and quantified by high performance liquid chromatography (HPLC). Effects on inflammation were analyzed by nutrigenomics using a human cell-based TPA-induced monocyte–macrophage differentiation model employing U-937 cells and inflammatory surrogate genes. Dose response and kinetics analysis of OPEs with different chemical profiles revealed less cytotoxic effects of PMFs as compared to OH-PMFs as demonstrated by the MTT-method. Noteworthy, a comparison of two PMF members such as 3,5,6,7,3′,4′-hexamethoxyflavone (HexaMF) and 3,5,6,7,8,3′,4′-heptamethoxyflavone (HeptaMF) exhibited less cytotoxic effects of HeptaMF as compared to HexaMF. A specific OPE enriched with HeptaMF, PMFs and OH-PMFs at low concentrations (10 μg/mL) significantly down-regulated the expression of a panel of genes involved in inflammatory response, including *COX-2*, *TNF-α*, *ICAM-1*, *NFκB*, *IL-1β*, *IL-6*, and *IL-8* with an inflammatory index of −0.55. The strong anti-inflammatory effects were then validated in a mouse carrageenan-induced paw edema model. Oral intake of OPE reduced paw edema significantly in a dose-dependent manner. Importantly, a dosage of 250 mg/kg gave an anti-inflammatory effect comparable to ibuprofen. A preliminary clinical study showed that OPE was well tolerated showing no adverse side effects. In summary, enrichment of phyto extracts such as OPEs with specific polymethoxyflavones as anti-inflammatory bioactives is a promising strategy to find naturally derived extracts that are effective against diseases associated with inflammation.

Keywords: Citrus flavonoids; Edema; Gene expression; Inflammation; Nutrigenomics

1. Introduction

Chronic inflammation is widely recognized as a major underlying cause of various degenerative diseases including cardiovascular, Alzheimer's, diabetes, and cancer [1–5]. In contrast, acute inflammation is a beneficiary response by promoting vasodilatation which enables rolling, adhesion, and endothelial transmigration of leukocytes toward infected tissue. Acute inflammation is initiated by the activation of a variety of inflammatory genes encoding for adhesion molecules (e.g. *ICAM-1*, *VCAM-1*), chemokines (e.g. *IL-8*), and metabolites generated in the arachidonic acid (AA) pathway [1,2,6]. Phospholipase A_2 provides AA as a substrate for cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) which generate a variety of prostaglandins and leukotrienes, respectively, triggering chemotaxis and vasodilation [1,2,6]. Activation of neutrophils enables phagocytosis and intracellular degradation of the ingested material mediated through lysosomal enzymes and oxidative burst. Oxidative burst is characterized by enzymatic generation of electrophilic species (ES) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by NADPH oxidase, myeloperoxidase and inducible nitric oxide synthase (iNOS). Chronic inflammation is characterized by prolonged duration of persistent infections, immune-mediated inflammatory diseases, or prolonged exposure to toxic reagents. Monocytes differentiation to macrophages is an important event in chronic inflammation. Macrophages as the dominant cellular

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player activate several cytokines (e.g. *IFN-γ*, *TNF-α*, *IL-1β*, and *IL-12*) and chemokines (e.g. *IL-8*, monocyte chemotactic protein-1, and macrophage inflammatory protein-1) to perpetuate the inflammatory response [1–3,6,7]. Activation of the transcription factor NFκB plays a central role in the induction of many key inflammatory genes such as *PLA_2*, *COX-2*, *iNOS*, *ICAM-1*, *IL-1β*, *IL-6*, and *IL-8* [2,3,6,7]. The function and interplay of key pro-inflammatory mediators in the inflammation cascade as well as the balance between their up-regulation and down-regulation may ultimately determine the degree of inflammation [1,2,8]. It is generally believed that during severe chronic inflammation, accumulation of tissue destruction caused by ES coupled with damage induced by proteolytic metalloproteinases leads to pathological conditions of various diseases including cardiovascular, Alzheimer's, diabetes, and cancer [1–5].

The limitation of current anti-inflammatory therapies is widely acknowledged and evident in the continuous efforts in the pharmaceutical industry to develop drugs targeting specific steps in the inflammatory cascade. Natural products have the potential to fill this therapeutic gap addressing the complexity in the inflammatory cascade thereby reducing side effects and compensatory reactions requiring secondary treatment [8]. Orange peel is rich in flavonoids including methylated derivatives such as polymethoxyflavones (PMFs). PMFs have been shown to exhibit strong anti-inflammatory effects both at the level of gene expression and enzyme activity [9–16]. In addition, induction of apoptosis by PMFs-mediated calcium-signaling may attenuate inflammation [17]. Flavonoids are typically found throughout the whole fruit whereas PMFs are found exclusively in the peels of *Citrus* genus, particularly in the peels of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulate*). As the most abundant PMFs in orange peel extract (OPE), tangeretin and nobiletin have been demonstrated to have strong anti-inflammatory effects as indicated by inhibition of *PLA_2*, *COX-2*, *iNOS*, *TNF-α*, *15-LOX*, *IL-1β*, *IL-6*, and NADPH oxidase in different cell-based and animal models [10,12–16,18,19]. Down-regulation of inflammatory genes by PMFs corresponded to suppression of NFκB, AP-1, and CREB [18]. Noteworthy, strong anti-inflammatory activities were found for 3,5,6,7,8,3′,4′-heptamethoxyflavone [20]. Strong anti-inflammatory activities were found also for OH-PMFs derived from OPE such as 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 3′-demethylnobiletin (3′-dNob), 4′-demethylnobiletin (4′-dNob), and 3′,4′-didemethylnobiletin (3′,4′-dNob) which attenuated *iNOS*, *TNF-α*, and *COX-2* expression [10,15,21–23].

In view of the growing evidence of anti-inflammatory bioactives in orange peel extracts, we have prepared six different OPEs containing different concentrations of PMFs and OH-PMFs. Effects of different OPEs on cell viability by the MTT-method were evaluated and correlated to different chemical profiles. The nutrigenomic method [24,25] was used as measure for anti-inflammatory bioactivity using a subset of inflammatory surrogate genes in a human monocyte–macrophage differentiation model [8]. The OPE enriched with bioactive polymethoxyflavones showed strong anti-inflammatory effects as demonstrated in a cell-based human *in vitro* monocyte–macrophage differentiation model and a paw edema *in vivo* mouse model.

2. Materials and methods

2.1. Materials and chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD). Cell culture flasks, dishes, and 24-well plates were from Falcon (Becton-Dickinson, Franklin Lakes, NJ). For RNA isolation, RNeasy™ Total RNA Kit (Qiagen, Chatsworth, CA) was used. Oligo-dT, dNTPs and Superscript™ II reverse transcriptase were purchased from Invitrogen, Life Technologies (Grand Island, NY). *Taq*Man qPCR probes, primers and master mix were from Applied Biosystems, Life Technologies (Grand Island, NY). Other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Preparation and analysis of orange peel extracts

2.2.1. Preparation of OPEs

Different batches of sweet orange peel extract (OPE, from cold-pressed orange peel oil) were purchased previously from Florida Flavors Company (Lakeland, FL). The OPEs were further purified with a flash chromatography system on a silica gel column to remove majority of essential oils as previous described [26] to obtain OPE with high content of polymethoxyflavones, such as OPE-6 and the precursors of OPE-1 to OPE-5. The obtained OPE precursors were suspended in absolute ethanol and further treated with concentrated hydrochloric acid for certain period of time monitored with HPLC system to get the desired content of hydroxylated PMFs. The reaction mixture was then cooled and concentrated in vacuum. The residue was dissolved in ethyl acetate and washed with water, 1 mol/L sodium bicarbonate, water and brine. The organic layer was dried over aqueous sodium sulfate, filtered to collect liquid and concentrated to remove solvent. The resulting OPEs were lyophilized overnight to get final products (OPE-1 to OPE-5).

As an example, the purchased commercial OPE mixture (10 g) was dissolved in a mixture of methylene chloride (2 mL) and hexanes (2 mL) and loaded onto a 120 g pre-conditioned silica gel flash column. The isocratic solvent was 10% ethyl acetate and 90% hexanes and kept eluting for 30 min. Then, another isocratic solvent system with 85% of ethyl acetate and 15% of hexanes was introduced and kept eluting while collecting eluent as one fraction for another 30 min. OPE (8 g) without essential oils was obtained. To the obtained OPE 100 mg, 20 mL of anhydrous ethanol was added and followed by the addition of 1 mL concentrated hydrochloric acid. The mixture was heated to boiling and remained reflux for 16 h. The reaction mixture was cooled and concentrated in vacuum. The resulted residue was redissolved in ethyl acetate and washed with water, 1 mol/L sodium bicarbonate solution, water and brine. The organic layer was separated and dried over anhydrous sodium sulfate. After