Polymethoxyflavones as Food Factors for the Management of Inflammatory Diseases

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ABSTRACT

Recent researches have expanded the concept that inflammation is a critical component of human diseases. The possible mechanisms by which inflammation contribute to various conditions include increase of reactive species and inflammatory cytokines and growth factors leading to alteration of biomolecules, induction of genetic change, cellular dysfunction and enhancement of transformation or proliferation. Thus, targeting the inflammation is potential strategy for prevention of chronic diseases. Numerous epidemiological and laboratory studies suggest that polymethoxyflavones (PMFs) exhibit a broad spectrum of biological activities. Recently, we isolated and identified hydroxylated PMFs from citrus peels and have investigated their biological activities, including anti-inflammation and cancer chemopreventive property. We suggest that hydroxylated PMFs possess greater biological activity potency for chemoprevention on targeting inflammation.

Key words: citrus peel, polymethoxyflavones, inflammation, disease

NATURAL OCCURRENCE AND CHEMISTRY OF POLYMETHOXYFLAVONES

Citrus production worldwide in major citrus producing countries in 2007 was 115.7 million metric tons. Total citrus production in the United States was 10.0 million metric tons (National Agricultural Statistics Service). Around 34% of these products were used for juice production[(1)], yielding approximately 44% (4 - 5 billion lbs in the USA) of peels as by-products.

Medicinally, orange peel has been used in traditional medicine in some Asian countries for relieving stomach upset, skin inflammation and muscle pain. Flavonoids, consisting of mainly polymethoxyflavones (PMFs), terpenoids, such as limonene and linalool, and other volatile oils are the major ingredients of orange peel.

PMFs exist almost exclusively in *Citrus* genus, particularly in the peels of two citrus species, sweet orange (*C. sinensis*) and mandarin (*Citrus reticulata* Blanco). So far, about 30 PMFs have been isolated and identified from different tissues of the citrus plants[(1,2)]. The types and content of PMFs vary between different varieties of citrus species.

The structures of PMFs usually differ in the numbers, types or positions of substitution on the 2-phenyl-γ-pyrone skeleton. The general structures of PMFs are illustrated in Figure 1. Interestingly, the majority of the identified compounds are derivatives from the base structure of flavones, though two flavanones were identified from Dancy tangerine leaves[(3)]. Among those identified PMFs, tangeretin and nobiletin have been intensively studied because of their relatively ready availability and pharmacological properties.

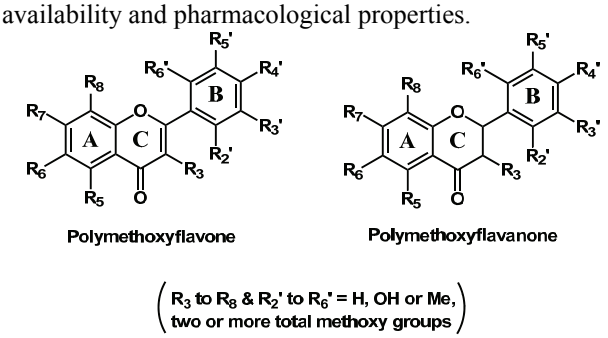

Figure 1. General structure of polymethoxyflavones (PMFs).

Recently, several 5-demethylated PMFs such as 5-demethylnobiletin, 5-demethyltangeretin, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone have been characterized in aged or long time storage orange peel and have led to a new research field because of their greater potency and better efficacy in biological activities than their PMF counterparts[(4,5)].

THE ROLE OF INFLAMMATION IN HUMAN DISEASES

Extensive research within past few decades has revealed the linked between inflammation and human diseases, including neurological diseases, cardiovascular

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diseases, obesity, metabolic disorders, bone and skeletal diseases, cancers and aging[6,7]. In normal condition, inflammation is a defense and protective mechanism that responses to tissue injury, bacteria infection, irradiation and wounding. Migration of immune cells is immediately occurred surrounding affected area. Inflammation triggering is initiated by the recognition of pathogen by immune cells of innate immune system[8]. These immune cells produce and release a series of mediators to amplify inflammatory signals, and consequently recruitment of inflammatory cells. Subsequently, recruited inflammatory cells release reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines to eliminate foreign bacteria and repair damaged tissue, and finally restoration of homeostasis that coordinate restitute by different cell types[8,9]. This inflammatory process is rapid and self-limiting that resolves by removal of inflammatory cells through programmed cell death or apoptosis. In general, inflammation is helpful to host defense and health. However, aberrant regulation and prolonged inflammation has found to contribute to the development of chronic inflammatory conditions[10].

It has been known that chronic inflammation is a critical driver of human diseases. Although chronic inflammation is not the major cause of disease but contributes to their pathogenesis. Serious tissue damage is a hallmark of chronic inflammation caused by over-activation of immune system, inefficient regulation and prolonged inflammatory state. During chronic inflammatory response, infiltrated immune and inflammatory cells produce a wide variety of free radicals, ROS and RNS including O_2-• (superoxide anion), •OH (hydroxyl radical), H_2O_2 (hydrogen peroxide) and nitric oxide (NO) that react chemically with diverse biomolecules, result in the function loss of genes, proteins, enzymes, and plasma membrane, ultimately cellular toxicity in surrounding tissues[7]. In addition, inflammatory signals also trigger a number of signaling cascades, gene expression and activation of enzymes in immune or inflammatory cells which in turn promote generation of various oxidants and proinflammatory molecules involved in expand tissue inflammation[7,9].

In cardiovascular disease, leucocytes adhesion and increase of oxidative stress damage endothelia cells, leading to dysfunction of vascular endothelium. Macrophage plays a pivotal role in pathogenesis of atherosclerosis. In early stage of atherosclerosis, increased adhesive property between monocyte and vascular endothelial cells through expression of adhesion molecules results in monocyte transmigrating into arterial intima and differentiating to macrophages, which uptake oxidized-LDL (oxLDL) to generate foam cells, and finally the formation of fatty streak[11]. This multiple process is triggered by different adhesion molecules, inflammatory cytokines, chemoattractant molecules and growth factor, including selectins, vascular cell adhesion molecule-1 (VCAM-1), interleukins (ILs), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and platelet-derived growth factor (PDGF)[6,11].

Activation of microglial and astrocytes, both immune cells in brain and central nervous system are the major cause of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[12]. Aggregated peptide (amyloid β peptide) and protein (α-synuclein protein) trigger activation of microglial and astrocytes that cause neuronal damage or neurotoxicity through up-regulation of ROS and various proinflammatory mediators including NO, prostaglandin E_2 (PGE_2), ILs and TNF-α. These inflammatory mediators produced by microglial and astrocytes also activate each other to amplify inflammatory state in neurons and promote neuropathology[6,12].

Obesity is a chronic inflammatory disorder supported by release of TNF-α and ILs from adipocyte that influences the function of fat tissue and recruitment of immune and inflammatory cells in adipose tissue, results in creation of a chronic low-grade inflammation by changing the morphology and composition of adipose tissue[13]. Increased TNF-α impairs insulin/IGF signaling and decrease of glucose uptake and expression of GLUT4 that leads to insulin resistance, which is a characteristic in most metabolic syndrome[13,14]. In osteoporosis, dysregulated inflammation from recruitment of immune cells produces excessive proinflammatory cytokines causing activation and differentiation of osteoclast, and decreases osteoblastogenesis that impairs bone remodeling[15].

Recently, numerous studies have indicated inflammation is one of major risk factors for aging and age-related diseases[16]. Although the detailed mechanism of inflammation and aging has not been understood, some hypotheses are proposed. During aging, the imbalance of redox status resulted from increased oxidative stress is crucial in regulation of inflammatory mediators expression, such as IL-1β, IL-6, TNF-α, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and lipooxygenase (LPO). Elevated levels of inflammatory cytokines are found in elderly people and patients with age-related diseases[17]. Activated pro-inflammatory signaling in aging is regulated by complicated mechanism including protein tyrosine kinase (PTK), mitogen activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB). It has been accepted that age-related oxidative stress-induced redox imbalance leading to systemic inflammation and contributes to pathogenesis of age-related diseases[16].

Growing evidences demonstrate that inflammation is a one of the hallmarker of cancer development. Early in 18th century, Virchow observed that cancers are frequently occurred at sites of chronic irritation[18]. Nowadays, numbers of evidence strongly supports that the notion of inflammation plays a central role in tumorigenesis of various cancers[19]. Inflammation is involved in every stage of cancer development, including initiation, promotion and progression. In response to

inflammatory stimulation, inflammatory cells are recruited in injury tissue and produce excessive reactive species (ROS and RNS) and pro-inflammatory cytokines to against infection. However, elevated ROS, RNS and cytokines also cause damage of normal tissue. Once surviving, the injured cells with genetic change, termed as initiated cells, tend toward proliferate and transform to malignant cells. As tumor tissue formation, cancer cells also produce chemokines to attract inflammatory cells and stromal cells, creating an inflammatory microenvironment that functions to support nutrition, growth factors and cytokines, and finally facilitate tumor growth and development. A lot of molecules are involved in inflammation-medicated tumorigenesis, including transcription factors (NF-κB and STAT3), inflammatory cytokines and enzymes (iNOS, COX-2, ILs, TNF-α), angiogenenic factor (HIF-1a and VEGF) as well as multiple upstream signaling pathways (PKC, MAPKs, PI3K/Akt)[6,19].

ANTI-INFLAMMATORY ACTIVITY OF POLYMETHOXYLFLAVONES

Numbers of studies suggest that PMFs in citrus peel exhibit a broad spectrum of biological activity, including anti-inflammatory, anti-carcinogenic, and anti-atherogenic properties. Previously, different hydroxylated PMFs and PMFs have been isolated from sweet orange (*Citrus sinensis*) peel[4]. We found 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OH-HxMF) demonstrated potent anti-proliferative activity through the induction of apoptosis in human leukemia cancer cells[4]. Moreover, 5-OH-HxMF treatment resulted in ROS production, triggered apoptosis signaling through mitochondria cytochrome c release and caspases cascade[5].

As mentioned before, inflammation is associated with the development of cancer[6,19]. Therefore, we investigated the anti-inflammatory property of 5-OH-HxMF by using *in vivo* mouse skin model. The mouse skin model has been extensively used to study multistep tumorigenesis and the molecular mechanisms. In this model, the characteristic of initiation stage is genetic damage in the formation of DNA adducts or initiator-induced DNA base changes, such as 7,12-dimethyl-benzanthracene (DMBA). The promotion stage of mouse skin carcinogenesis involves the production and maintenance of a chronic state of hyperplasia and cell proliferation and ultimately the selective clonal expansion of initiated cells. The classical skin tumor promoter is tetradecanoylphorbol-13-acetate (TPA), and is known to up-regulation of arachidonic acid cascade and ornithine decarboxylase (ODC) in mouse epidermis. Indeed, TPA treatment induces a reaction similar to the inflammatory wound reaction by enhancing the expression of a wide variety of proinflammatory mediators and growth factors that leading to recruit inflammatory cells and promote tumor cell proliferation[20]. In our study, we found that topical application of TPA in mouse skin caused leukocytes infiltration and epidermal proliferation. Pre-treatment with 5-OH-HxMF (1 and 3 mol) resulted in significantly inhibited TPA-induced mouse skin inflammation by decreasing inflammatory parameters[21]. At molecular levels, pre-treatment with 5-OH-HxMF reduced TPA-induced both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression through interfering with MAPKs, PI3K/Akt and PKC signaling, leading to blocking the activation of transcription factors (NF-κB and STAT3). Furthermore, 5-OH-HxMF significantly inhibited DMBA/TPA-induced skin tumor formation by reducing the tumor incidence and tumor multiplicity of papillomas at 20 weeks[21]. By measurement of the diameters of skin tumors, we also found the tumor size in 5-OH-HxMF treated group tended to be smaller than that of untreated group, which might contribute to decreased iNOS, COX-2 and VEGF protein levels.

Nobiletin is a major PMF in citrus fruits (particularly in the peel). Both *in vitro* and *in vivo* data have shown that nobiletin has anti-inflammatory and anti-carcinogenic activities. The biotransformation study of nobiletin has shown that it undergoes demethylation pathway with the formation of mono-demethylated nobiletin as major metabolites. We have identified two known nobiletin metabolites in mouse urine, 3′-demethylnobiletin and 4′-demethylnobiletin as well as a novel one, 3′,4′-didemethylnobiletin (DDMN)[22]. In lipopolysaccharide (LPS)-stimulated macrophage, co-treatment with three metabolites all markedly reduced LPS-induced nitrite production. Among them, DDMN significantly inhibited both iNOS and COX-2 gene expression in LPS-stimulated RAW264.7 macrophage. Furthermore, we compared the anti-inflammatory activity of nobiletin and its metabolite DDMN in mouse skin. Our data showed that application of DDMN before TPA treatment afforded significant inhibition of TPA-induced iNOS, COX-2 and ODC expression in a dose-dependent manner[23]. DDMN inhibited TPA-induced DNA-binding activity of NF-κB and AP-1 by suppressing phosphorylation of IκBα膦and p65 and subsequent nuclear translocation of p50 and p65/RelA subunits of NF-κB and PKC activity. More importantly, DDMN showed markedly more strong anti-tumor promoting effect than nobiletin according to the reduced tumor number[23]. Taken together, above results demonstrated that the metabolite, DDMN, possess more potent anti-inflammatory activity than its parent compound.

Colorectal cancer (CRC) is one of major causes of cancer-related mortality in both men and women worldwide. Inflammation plays a major role in pathology of colon cancer[24]. Pervious studies showed that feeding nobiletin reduced carcinogen azoxymethane (AOM)-induced aberrant crypt foci (ACF) formation in rats[25]. More recently, we found the potent inhibitory

effects of 5-hydroxy PMFs (5-hydroxy-6,7,8,3',4'-pentamethoxyflavone, 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone, and 5-hydroxy-6,7,8,4'-tetramethoxyflavone) compared to their permethoxylated counterparts on human colon cancer HCT116 and HT29 cells[26]. In addition, dietary hydroxylated PMFs (0.01 and 0.05%) strongly reduced numbers of large aberrant crypt foci (≧ 6 ACF) and tumors in colonic tissue in male ICR mice[27]. Dietary hydroxylated PMFs interfered with Wnt/β-catenin and epidermal growth factor receptor (EGFR)/Ras/MAPKs pathways, blocked activation of NF-κB and STAT3 that consequently decreased the expression of iNOS, COX-2, cyclin D1 and VEGF, resulting in suppression of colonic tumorigenesis[27]. Overall, these evidences suggested that hydroxylated PMFs have great potential as a novel chemopreventive agent to be used in the treatment of inflammation associated with tumorigenesis.

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