REVIEW

The importance of natural product characterization in studies of their anti-inflammatory activity

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The knowledge that natural products provide a rich source for therapeutic discovery has led to the development of many of the world's most commonly used drugs. In view of the growing need for effective anti-inflammatory agents, the potential for natural products to serve as safe and effective therapeutic agents has gained increasing attention. However, polymolecular extracts must be rigorously evaluated and chemically characterized to insure adequate consistency in performance. The research in this field has been plagued by inconsistencies due in part to inadequate chemical characterization and documentation, making comparison of results across studies very difficult. Analytical chemistry and molecular methods now exist to insure sufficient transparency to avoid this limitation. Further, our understanding of the complexity of inflammation has advanced to enable significant insight into the mechanism of action of these natural extracts. Here, we review the inflammatory pathways targeted by many therapeutic agents, discuss the value of natural products as anti-inflammatory agents, review approaches for their biological and chemical evaluation, and highlight challenges to the field. We present two examples highlighting the rigorous use of cell, molecular, and chemical methods for characterization and quality control as templates for future studies of anti-inflammatory activity of natural products.

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1 Inflammation and natural products

1.1 Acute and chronic inflammation

Acute inflammation is an essential and complex response protecting the body against harmful stimuli such as pathogens, damaged cells, or other irritants, and is manifested by vascular changes, edema, and predominantly neutrophilic infiltration in a matter of days (for overview, see [1, 2]). Vasodilation of blood vessels and increased vascular permeability is elicited by inflammatory mediators (nitric oxide (NO), histamine, serotonin, bradykinin, prostaglandins, leukotrienes, platelet-activating factor, and substance P). This process promotes and enables rolling, adhesion, and endothelial transmigration of leukocytes (*e.g.* neutrophil granulocytes) toward the site of tissue infection. Extravasation of neutrophils is coordinated by

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Abbreviations: AA, arachidonic acid; **AP-1,** activator protein-1; **BTE,** black tea extract; **CF,** caffeine; **COX-2,** cyclooxygenase-2; **CPE,** citrus peel extract; **CREB,** cyclic-AMP-response element binding protein; **ECG,** epicatechin gallate; **EGC,** epigallocatechin; **EGCG,** EGC gallate; **ES,** electrophilic species; **GA,** gallic acid; **GM-CSF,** granulocyte macrophage colony-stimulating factor; **ICAM-1,** intercellular adhesion molecule-1; **iNOS,** inducible nitric oxide synthase; **5-LOX,** 5-lipoxygenase; **NFκB,** nuclear factor κB; **NO,** nitric oxide; **OPE,** orange peel extracts; **PLA_2,** phospholipase A_2; **PMF,** polymethoxyflavones; **ROS,** reactive oxygen species; **TF1,** theaflavin; **TF2a,** theaflavin-3-gallate; **TF2b,** TF-3′-gallate; **TF3,** theaflavin-3,3′-digallate; **TNF-α,** tumor necrosis factor-α

adhesion molecules (intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)), chemokines (*e.g.* IL-8), and metabolites generated in the arachidonic acid (AA) pathway [2, 3]. Phospholipase A_2 (PLA_2) provides AA as substrate for cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) which generate a variety of prostaglandins or leukotrienes, respectively, triggering chemotaxis and vasodilation [2]. The *COX-2* gene has been an attractive target for anti-inflammatory therapy since it is highly inducible and regulated by different transcription factors such as nuclear factor κ B (NFκB), activator protein-1 (AP-1), cyclic-AMP-response element binding protein (CREB), nuclear factor IL-6, or CCAAT/enhancer-binding protein [2, 4, 5]. In the LOX pathway LTA_4 hydrolase is particularly important, generating the potent neutrophil chemoattractant leukotriene B_4 [2, 6].

Activation of neutrophils *via* Toll-like-, G protein-coupled-, cytokine-, or phagocytic receptors 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 generated by NADPH oxidase or myeloperoxidase. The inducible nitric oxide synthase (iNOS) also participates in oxidative burst by NO generation which – aside from its role as a vasodilator – gives rise to the highly toxic peroxynitrite, in combination with superoxide anion radicals produced by NADPH oxidase [7, 8]. Usually, the acute inflammatory response is terminated within days after the inflammatory stimulus declines due to the short half-life of inflammatory mediators and neutrophils and the liberation of anti-inflammatory cytokines (*e.g.* transforming growth factor-β and IL-10) or mediators (*e.g.* lipoxins, resolvins, and protectins).

Chronic inflammation is characterized by prolonged duration (weeks or months) caused by persistent infections, immune-mediated inflammatory diseases, or prolonged exposure to toxic reagents. This results in severe tissue destruction caused predominantly by mononuclear macrophages. Macrophages are the dominant cellular player in chronic inflammation, with a life span of several months to years. Macrophages differentiate from monocytes (half life about 1 day) when they cross the endothelium, a process governed by adhesion molecules (*e.g.* ICAM-1 and VCAM-1) and chemokines (*e.g.* monocyte chemotactic protein-1 and macrophage inflammatory protein-1). Noteworthy, there is bidirectional positive interaction between lymphocytes and macrophages during chronic inflammation. This feed forward interaction involves several cytokines (*e.g.* IFN-γ, tumor necrosis factor-α (TNF-α), IL1-β, and IL-12) and chemokines (IL-8, monocyte chemotactic protein-1, and macrophage inflammatory protein-1), and acts to perpetuate the inflammatory response [1, 2, 5]. The activation of macrophages is induced mainly *via* Toll-like receptor-signaling and IFN-γ liberated by activated T-lymphocytes. In addition, a variety of ILs (IL-12, -15, and -18) increase IFN-γ expression, leading to macrophage activation and oxidative burst [2, 8, 9].

1.2 Inflammatory pathways

The function and interplay of pro-inflammatory mediators in the inflammation cascade activated through free radical-induced, receptor-dependent extrinsic or intrinsic mechanisms is shown in Fig. 1. The balance between upregulation and downregulation and the interrelationship between key inflammatory mediators may ultimately determine the degree of inflammation. On a molecular level, cytokines such as TNF-α and IL-1β are critical in initiating the inflammatory response by binding to their receptors and activating NFκB through distinct pathways [2, 10]. The *trans*-acting *cis*-responsive factor NFκB is highly regulated through multiple pathways and through interaction within clusters of other transcription factors. These regulatory networks control the induction of many key inflammatory genes such as PLA_2, COX-2, 2-LOX, iNOS, ICAM-1, IL-1β, IL-6, IL-8, IFN-β, and granulocyte macrophage colony-stimulating factor (GM-CSF). Activation of NFκB thus plays a central role to initiate and promote the inflammatory response [1, 2, 5, 10–12]. AP-1, another transcription factor for many inflammatory genes, is controlled by different compositions of homodimers and/or heterodimers of the Jun, Fos, activating transcription factor, and musculoaponeurotic fibrosarcoma protein families [13]. CREB appears to play a main role in the induction cascade as transcriptional coactivator known to interact with a variety of transcription factors. Crosstalk among NFκB, AP-1, and CREB has been suggested, creating an additional layer of regulatory hierarchy [10, 14–17].

1.3 Chronic inflammation and disease

Chronic inflammation is widely recognized as the major underlying cause of six of the top ten causes of death in US, including cardio and cerebrovascular disease, autoimmune disease, chronic respiratory disorders, Alzheimer's disease, cancer, and diabetes [2, 12, 18–20], accounting for millions of dollars of healthcare costs and human suffering. It is generally believed that accumulation of tissue destruction caused by ES together with proteolytic metalloproteinases during chronic inflammation lead to pathological conditions of various diseases [1, 2, 5, 8, 12, 21–25]. For instance, in diabetes, reactive carbonyl species formed as a result of hyperglycemia trigger inflammation and pathology directly as well as indirectly through reaction with proteins such as hemoglobin and proteins within the endothelium causing impaired oxygen transport and vascular elasticity contributing to insulin resistance [26, 27]. Current anti-diabetic drugs, even when they successfully control blood glucose, fail to contain the inflammatory processes which contribute

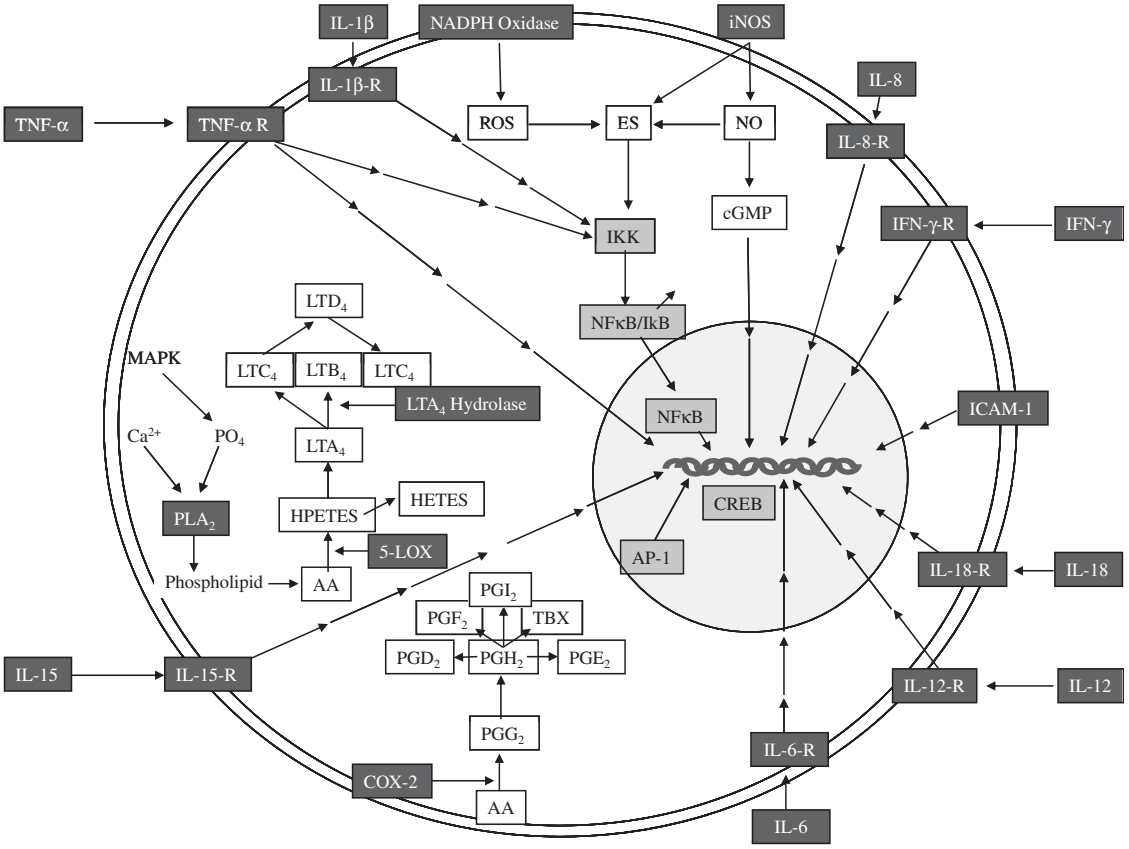

Figure 1. Inflammatory pathways in human cells. The network of key mediators for inflammation is shown. Inflammatory effectors and receptors (black boxes), transcription factors and cofactors (grey boxes), or other mediators (white boxes) are shown. Briefly, transmigration of leukocytes toward the site of tissue infection is triggered by ICAM-1 and IL-8. PLA_2 cleaves AA from phospholipids as substrate for COX-2 and 5-LOX. Conversion of AA to prostaglandins and thromboxanes (COX-2) and leukotrienes (5-LOX) induces chemotaxis, vasodilatation, and fever. LTA_4 hydrolase generates the potent chemoattractant LTB_4. TNF-α and IL-1β are critical in initiating the inflammatory response by binding to their respective receptors and inducing fever, vasodilatation, and stimulation of T-lymphocytes or apoptosis. Expression of inflammatory genes is triggered by NFκB (binding sites in promoters of PLA_2, COX-2, 2-LOX, iNOS, ICAM-1, IL-1β, IL-6, IL-8, IFN-β, and GM-CSF) and AP-1 (binding sites in promoters of COX-2, iNOS, ICAM-1, TNF-α, IL-1β, IL-6, IL-8). CREB has a main role as transcriptional coactivator of NFκB and AP-1. IFN-γ activates NFκB and induces macrophage activation and apoptosis but also has anti-inflammatory properties (induces expression of IL-1 receptor antagonist, downregulates IL-1 and IL-8). Generation of pro-inflammatory ES is mediated by NADPH oxidase (*e.g.* O_2^-) and iNOS (*e.g.* NO). The other ILs have pleiotropic functions such as fever induction, T-lymphocyte stimulation, downregulation of TNF-α and IL-1β (IL-6), IFN-γ induction and release, regulation of T(H1) cells (IL-12), activation of T cells and macrophages, STAT signaling, TNF-α and IL-1β induction (IL-15), and expression of IFN-γ, TNF-α, IL-1β, GM-CSF, and IL-8 (IL-18). Abbreviations: 5-LOX (5-lipoxygenase), AA (arachidonic acid), AP-1 (activator protein-1), COX-2 (cyclooxygenase-2), CREB (cAMP-responsive element binding protein), ES (electrophilic species), HPETES (hydroperoxyeicosatetraenoic acids), HETES (hydroxyeicosatetraenoic acids), ICAM-1 (intercellular adhesion molecule-1), iNOS (inducible nitric oxide synthase), iκB (inhibitory subunit of NFκB), IKK (IκB kinase), LT (leukotriene), MAPK (mitogen-activated protein kinase), NFκB (nuclear factor kappa B), NO (nitric oxide), PLA_2 (phospholipase A_2), PGs (prostaglandins), ROS (reactive oxygen species), TBX (thromboxane), TNF-α (tumor necrosis factor-α), and TNF-α R (TNF-α receptor).

to organ pathology [28]. Aging increases the risk of most of these inflammation-related diseases, and this association has been attributed to an age-dependent accumulation of ROS-induced cell damage [2, 18, 20, 29]. The limitation of current anti-inflammatory therapies is widely acknowledged and evident in the continued efforts of the pharmaceutical industry to develop drugs targeting specific steps in the inflammatory cascade. We propose that natural products have the potential to fill this therapeutic gap in a way that acknowledges the complexity of the inflammatory cascade, whereas reducing the potential for side effects and compensatory reactions requiring secondary treatment.

1.4 Natural products as anti-inflammatory agents

1.4.1 Historical use

Natural products have been used as medicinal products for centuries globally although the sources and applications

vary among different regions. Hippocrates, the father of modern medicine left historical records of pain-relief treatments, including the use of powder made from the bark and leaves of the willow tree to help heal headaches, pains, and fevers. Not until 1829, by means of isolation and characterization, scientists discover that the function of salicin in willow plants was the pain reliever and further developed the most popular drug, aspirin. Hippocrates' statement to ''Let food be thy medicine and medicine thy food'' presaged the modern science of therapeutic nutrition and development of nutraceuticals and medical foods by over two millennia. Not until the development of modern chemical methods, it has been possible to identify and characterize the nutrients and phytochemical bioactive compounds such as polyphenols in plants whose therapeutic properties have been relied upon throughout history [30].

1.4.2 Therapeutic potential

The knowledge that natural products provide a rich source for therapeutic discovery has led to the development of many of the world's most commonly used drugs. For instance, from 1940 to July 2006, among 155 available small molecular anti-cancer drugs, 113 (73%) of them directly came from or were inspired by natural products [30]. Although single pure chemical compounds can be very potent therapeutic agents, adverse side effects often lead to poor compliance, limited applications, and potential withdrawal from the market. Consumer perception is growing that the use of natural bioactive products more closely resemble their natural state (*e.g.* extracts rather than isolated single compounds) and may elicit fewer side effects and be similarly or more effective than new chemical entities. It is possible that consuming bioactive compounds as part of a complex mixture – *i.e.* in their native chemical environment – reduces the potential for adverse effects as different components may influence absorption, metabolism, and excretion of a primary bioactive. Additionally, compounds of complex natural extracts may act synergistically in ways that are difficult to reveal experimentally and produce effects that are unlikely to be replicated with single pure compounds. In addition, recent data from many laboratories suggest that natural products tend to act more subtly and broadly than most drugs, with modest effects at multiple steps in the inflammatory cascade rather than exerting potent action at a single step, as drugs are designed to do.

1.4.3 Challenges and open questions

Despite the potential advantages of natural products as therapeutic agents, this approach presents several significant challenges which have slowed development and acceptance by the mainstream medical enterprise. First, highly specialized and costly analytical instrumentation and techniques are required to characterize the bioactive components of a polymolecular composition, and to synthesize the standards needed for effective identification and quality control. For instance, hundreds of studies have been performed using green and black teas and their extracts, yet many of these studies failed to provide adequate compositional data, and sufficient information regarding the analytical methods used to characterize the composition used. Although some publications provide specific content (caffeine (CF), total polyphenols, EGC gallate (EGCG), and theaflavins (TFs)), in the absence of a full chromatogram, with methodological details, replication of the study material can be difficult. This has contributed to inconsistencies in the literature in this field. Second, delineating the mechanism of anti-inflammatory action requires the use of bioassays and molecular techniques that only recently have achieved a high level of reliability, cost efficiency, and throughput. Bioassays are essential in evaluating the consistency of anti-inflammatory activity; they can be very sensitive to chemical differences even in the absence of knowledge regarding the identity of minor chemical components. Third, reconstituting the biological activity of the extract from a combination of individual components has proven difficult, raising the possibility that minor or unidentified components act synergistically or antagonistically to influence bioactivity, or the components must be present within the biochemical milieu of the extract to function properly. In the absence of successful reconstitution from single, well-characterized chemical entities, the challenge is to devise adequate controls for chemical and biological quality assurance of a complex mixture. Such a complex material, like a food or flavor, is likely to contain a large number of compounds, many of which are present at extremely low levels, and may be unstable during analytical procedures. We propose a combination of biological and chemical assay methods to adequately insure quality control of natural products to achieve a level of precision and reliability needed for use in medical food or other therapeutic nutrition applications.

2 Strategies and challenges in chemical characterization of natural products

The chemical characterization of natural products presents several challenges. First, natural products used historically for therapeutic purposes typically were generated as fermentations, extracts, concentrates, or teas in liquid or dried form. These processes, even where clear documentation exists, are often variable and difficult to control, and can generate distinct chemical profiles due to subtle variations in processing technique. Second, even a ''simple'' extract may be composed of hundreds or even thousands of individual components with varying stabilities and potentials for interactions. Fortunately, modern analytical methods such as LC-MS-MS help to disentangle complex compounds, and

techniques exist for isolation and identification of virtually any chemical class. The application of these techniques, in combination with cell and molecular bioassays, enables identification of key bioactive components. Therefore, obstacles to accomplishing meaningfully defined or standard controlled natural product research will be (i) the isolation, characterization, and biological screening of natural products and (ii) the application of these chemically defined and biologically confirmed components as standardized controls in natural product research.

2.1 Factors contributing to variability

Another challenge to the development of natural products such as medical foods and therapeutics is created by the inherent variability in the starting material – whether a freshly harvested plant or a dried or partially processed plant product. There are several factors that contribute to this variability: (i) species, (ii) growing locations and conditions, (iii) processing and storage conditions, and (iv) final processed forms of natural products. Again, studies reporting therapeutic effects often fail to provide botanical species and these names can even be misused by the community. Inadequate documentation of each of these factors can make it impossible to replicate a study even within the same laboratory.

2.2 Technical advances enabling improved analytical characterization

Due to the rapid development of computer, information, and material technology in the last two decades, the technical capability of natural product characterization has advanced dramatically. Advanced qualitative and quantitative analytical instruments include HPLC, ultra-HPLC, HPLC/MS/MS, and HPLC/NMR. The LC/MS/MS exemplifies the dramatic advancement that has occurred; the application of LC/MS/MS in analysis and chemical structure determination can shorten the characterization time from weeks or months to hours.

2.3 Reference materials and standard protocols

The initial identification of natural product molecules involves multiple steps: extraction, isolation, and characterization. Extracted compounds from the initial identification process are treated as standards or benchmarks for natural product research. Standards of the highest purity possible must be used as reference molecules, and serve as a necessary quality control component for the development of analysis protocols in the research of natural products using HPLC, LC/MS, LC/MS/MS, *etc*. However, adequate quality control processes have been applied sparsely in most natural product research, and there has often been a dissociation between the content listed and the biological activity of the natural product.

3 Examples: characterization of anti-inflammatory bioactive compounds from two widely consumed natural products

3.1 Polyphenols from black tea

Catechins (*e.g.* EGCG) and TFs derived from green or black tea appear to be the primary bioactive compounds in suppression of key cytokines in the inflammation cascade as demonstrated by various cell-based and animal studies [31–36]. For our studies, we used a black tea extract (BTE) characterized by HPLC analysis. Nine compounds have been quantified such as CF, epicatechin, epigallocatechin (EGC), epicatechin gallate (ECG), EGCG, TF1, TF2-gallate

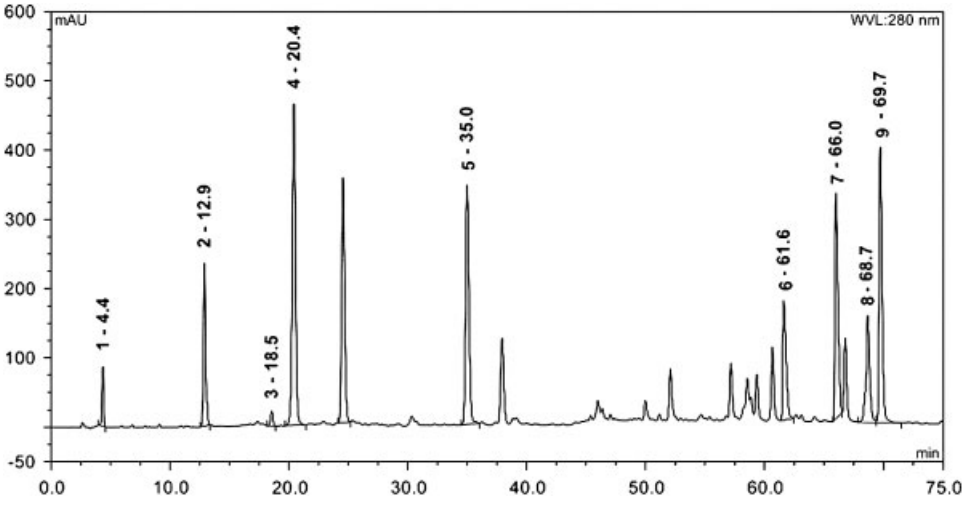

Figure 2. Typical HPLC diagram of BTE. HPLC conditions: Luna C18 column from Phenomex, 4.6 × 150 mm, 3 μm, 100 A; UV 280 nm; mobile phase A, water/0.1% HOAc, mobile phase B, acetonitrile; flow rate, 0.8 mL/min. Peaks: 1, GA; 2, CF; 3, EGC; 4, EGCG; 5, ECG; 6, TF1; 7, TF2a; 8, TF2b; 9, TF3. All 12 standard compounds (CF, epicatechin, EGC, ECG, EGCG, TF1, TF2a, TF2b, TF3, GA, theobromine, and theophylline) were either purchased from commercial source or isolated in-house and characterized by HPLC, MS, and NMR prior to use.

(TF2a), TF2-gallate (TF2b), and TF3,3′-digallate (TF3). For each batch of BTE, close attention is also given to the amount of gallic acid (GA), theobromine, and theophylline as well as abnormal peaks occurring in the HPLC profile. Figure 2 is a typical HPLC trace of BTE used as a routine method for quality control.

Using a human cell-based monocyte–macrophage differentiation model, we observed significant down-regulation of *COX-2*, *TNF-α*, *TNF-α* receptor, *ICAM-1*, *IL-1β*, *IL-6*, *IL-8*, *NFκB*, *C-JUN* and *p53* in response to BTE as demonstrated by cDNA and oligo microarray analysis (Fig. 3). In addition, whole genome analysis demonstrated an upregulation of anti-inflammatory *IL-10*, *ferritin*, *thioredoxin reductase*, and *heme oxygenase* (unpublished results). Downregulation of inflammatory genes correspond to attenuation of the canonical NFκB pathway through IκB kinase inhibition by catechins and TFs [37–39] and inhibition of AP-1 activity [39–41]. There is molecular evidence also for a role of CREB and nuclear factor IL-6 (CCAAT/enhancer-binding protein) in modulation of key *trans*-responsive inflammatory genes [16, 36]. Strong anti-oxidative properties of polyphenols in green and BTEs also seem to have a major anti-inflammatory effect since free radical-induced cell damage is one of the hallmarks in chronic inflammation [2, 5, 34, 35]. The modulation of other pathways such as G-protein signaling might add another potential anti-inflammatory mechanism elicited by tea bioactives [2, 31].

In other studies, we demonstrated that BTE reduced edema formation in a dose-dependent manner to the levels exerted by indomethacin and aspirin using different animal models (unpublished results); and tissue and blood analysis revealed a concomitant reduction in inflammatory mediators such as COX-2 and TNF-α [42, 43]. Moreover, small pilot clinical studies employing LPS-mediated irritation or exercise-induced inflammation showed prominent anti-inflammatory effects of BTE as indicated by a down-regulation of different cytokines and chemokines as well as

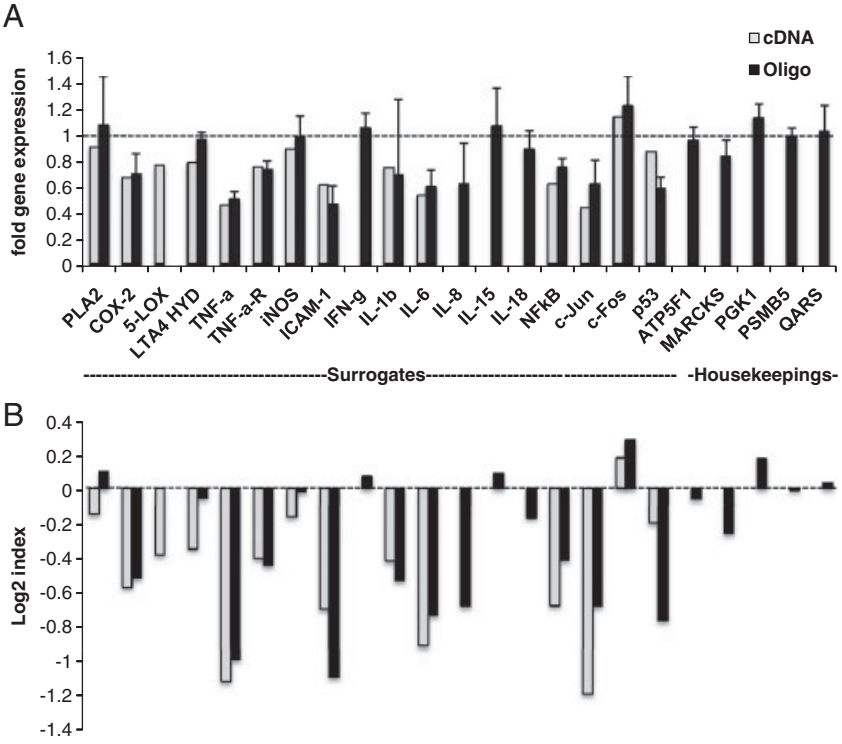

Figure 3. Effect of BTE on the expression of inflammatory genes in a human cell-based model. Human monocytes (U-937) were treated with TPA (20 nM) either alone or in combination with BTE (100 μg/mL) for 3 h. After RNA isolation and reverse transcription, expression of *PLA_2*, *COX-2*, *5-LOX*, *LTA_4 hydrolase*, *TNF-α*, *TNF-α receptor*, *iNOS*, *ICAM-1*, *IFN-γ*, *IL-1β*, *IL-6*, *IL-8*, *IL-15*, *IL-18*, *NFκB*, *c-Jun*, *c-Fos*, and *p53* was analyzed by proprietary cDNA (grey columns; one experiment) and oligo microarray (black columns; mean values+standard deviation of three independent experiments) analysis. As housekeeping genes, ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1 (*ATP5F1*), myristoylated alanine-rich protein kinase C substrate (*MARCKS*), phosphoglycerate kinase 1 (*PGK1*), proteasome (prosome, macropain) subunit, β type, 5 (*PSMB5*), and glutaminyl-tRNA synthetase (*QARS*) were employed. (A) Gene expression is expressed as ''fold gene expression'' after calculating the ratio of mean experimental channel (TPA+BTE) to the mean control channel (TPA alone) normalized to spike in controls. (B) Gene expression is shown as ''Log2 index,'' characterized by the average of Log2 of the ratios of all genes. Dotted lines indicate no changes in gene expression for ratio (A) or Log2 index (B), respectively. Log2 indices of −0.512 and −0.393 for cDNA and oligo array, respectively, indicate strong anti-inflammatory potential of BTE.

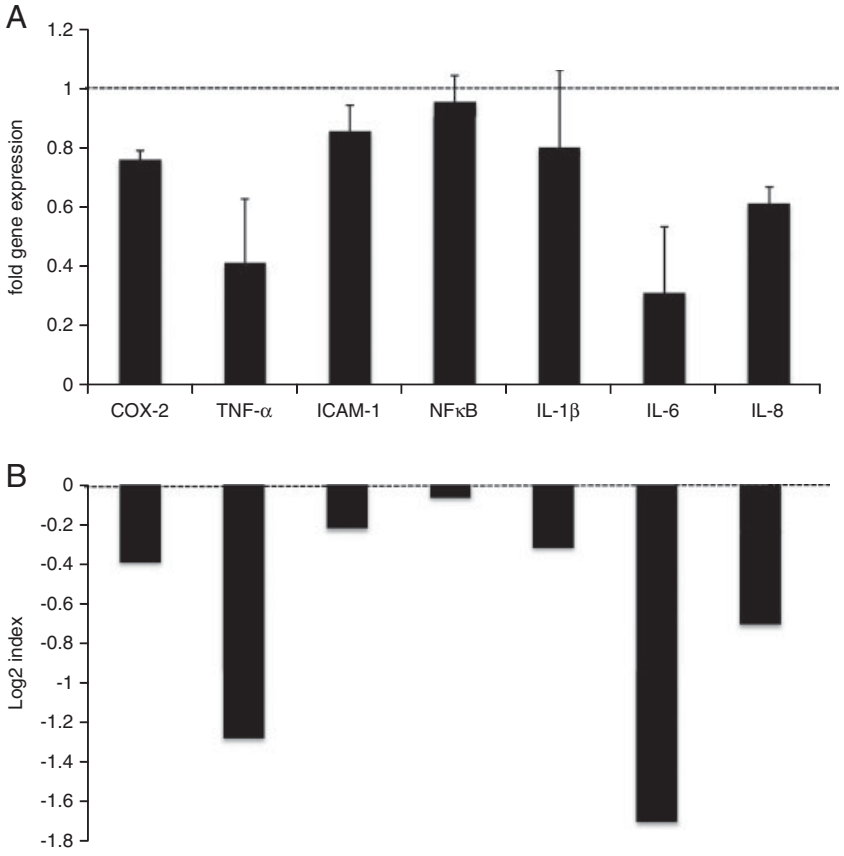

Figure 4. Effect of OPE on the expression of inflammatory genes in a human cell-based model. Human monocytes (U-937) were treated with TPA (20 nM) either alone or in combination with OPE (10 μg/mL) for 3 h. After RNA isolation and reverse transcription, gene expression of *COX-2*, *TNF-α*, *ICAM-1*, *NFκB*, *IL-1β*, *Il-6*, and *IL-8* was analyzed by TaqMan qPCR analysis. After normalization to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), gene expression was calculated according to the delta–delta CT method. (A) Gene expression is shown as the ratio of the mean experimental channel (TPA+OPE) to the mean control channel (TPA alone). Mean values+ standard deviation of three independent experiments are shown in the histograms. Significant downregulation ($p<0.05$) was found for *COX-2* and *IL-8*. (B) Gene expression is shown as '' Log2 index,'' characterized by the average of Log2 of the ratios of all seven genes. Dotted lines indicate no changes in gene expression for ratio (A) or Log2 index (B), respectively. Log2 index of −0.55 indicates strong anti-inflammatory potential of OPE.

reduced inflammatory symptoms such as ROS reduction and delayed onset muscle soreness [44, manuscript in preparation]. Our results of the anti-inflammatory effects throughout different cell-based assays, animals, and humans suggest that cell-based models to be predictive in the search for natural products and BTE to be very promising against diseases associated with inflammation.

3.2 Polymethoxyflavones from citrus peel

Polymethoxyflavones (PMFs) from citrus peel extract (CPE) exhibit anti-inflammatory activity both at the level of gene expression and at the level of enzyme activity [45–52]. In addition, induction of apoptosis by PMFs mediated *via* calcium signaling may attenuate inflammation [53, 54]. As the most abundant PMFs in CPE, tangeretin and nobiletin have been the focus of primary research examining their bioactivity. The majority of these studies demonstrate an 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 [45–52]. Similar to polyphenols derived from BTE (Section 3.1), downregulation of inflammatory genes by PMFs corresponded to the suppression of NFκB, AP-1, and CREB [46]. Noteworthy, anti-inflammatory activities were also found for other PMFs derived from CPE such as 3,5,6,7,8,3′,4′-heptamethoxyflavone, 3′-demethylnobiletin, 4′-demethylnobiletin, and 3′,4′-didemethylnobiletin which attenuated *iNOS* and *COX-2* expression [45, 50, 51].

The major compounds in orange peel extracts (OPE) have been isolated and characterized in our lab: sinensetin, nobiletin, tangeretin, tetra, hexa-, and hepta-MFs. In addition to these six PMFs, their 5-demethylated derivatives were also included as HPLC standards [55]. Employing our cell-based biological screening, we observed a downregulation of inflammatory genes such as *COX-2*, *TNF-α*, *ICAM-1*, *NFκB*, *IL-1β*, *IL-6*, and *IL-8* in response to OPE (Fig. 4) which correlated to a dose-related reduction in edema formation in a mouse model (unpublished results).

4 Concluding remarks

The reconstitution of biological activity from individual chemically pure components remains a challenge for natural products with therapeutic efficacy. Indeed, it is possible that the very complexity of the product is the ''special'' feature that provides such efficacy in the absence of undesired adverse side effects. In the absence of complete chemical specificity and control in a formulation, adequate characterization of polymolecular compositions must be considered to assure consistently reproducible biological activity. Biological assays

may be more sensitive to subtle changes in the chemical composition of natural products than highly sophisticated chemical methods used for quality control purposes. Thus, adequate quality control may ultimately require a combination of bioassay and chemical fingerprinting. The development of safe and effective therapeutic products derived from natural products is currently being held back by the perception and, occasional reality, of inadequately rigorous or poorly documented science. Publications investigating the biological impact of natural products must be held to the highest standards of methodological rigor, and improved standards for methods and analytical reference materials are needed for use by researchers and the industry alike. Only then, natural products will have the opportunity to be considered as safe and effective therapeutic options for inflammation-associated diseases.

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