In Vitro and in Vivo Anti-Inflammatory Activity of a Seed Preparation Containing Phenethylisothiocyanate

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Received October 3, 2005; accepted December 21, 2005

ABSTRACT

Winter cress (Barbarea verna) seed preparations rich in phenethylisothiocyanate (PEITC) had strong in vivo and in vitro anti-inflammatory activity, significantly reducing the size of carrageenan-induced rat paw edema. This in vivo effect was comparable with that of the nonsteroidal anti-inflammatory drug aspirin. The seed preparation, in a concentration-dependent manner, reduced the mRNA levels of inflammation-related genes such as the inducible forms of cyclooxygenase and nitric-oxide synthase and the proinflammatory cytokine interleukin in lipopolysaccharide-stimulated mouse macrophage cell line RAW 264.7. Activity of the seed preparation was similar to that of the synthetic PEITC. PEITC was the most active of five different forms of isothiocyanate tested for their effects on in vitro proinflammatory gene expression. In vivo activity of the seed preparation was also compared with that of two known anti-inflammatory drugs. We conclude that Barbarea verna seed preparation may function as a potent anti-inflammatory agent, interfering with the transcription of proinflammatory genes.

Phenethylisothiocyanate (PEITC) is an organosulfur bioactive compound present in many plants of the Brassicaceae family, most of which have long histories of human consumption. Of these, Barbarea verna (winter cress) is the richest source of PEITC (Ribnicky et al., 2001), with the highest levels formed in the seeds. Winter cress is an annual herb, mostly harvested as a seedling to add a peppery flavor to salads, sandwiches, soups, and garnishes. PEITC has many well-documented cancer chemopreventive properties. In addition, other related health-promoting effects in context of its anticarcinogenic activities, including anti-inflammatory, have been reported (Stoewsand, 1995; Heiss et al., 2001; Chen et al., 2003; Gerhauser et al., 2003; Rose et al., 2005).

Chronic inflammation, carcinogenesis, autoimmune disorders, and metabolic syndrome are mechanistically linked (Oshshima and Bartsch, 1994; Heiss et al., 2001; Esposito and Giugliano, 2004) and therefore may benefit from similar treatments. Chronic inflammation and infection lead to the up-regulation of enzymes and signaling proteins in affected tissues and cells. For example, proinflammatory enzyme inducible nitric-oxide synthase (iNOS) is involved in multiple sclerosis and Parkinson's and Alzheimer's diseases, as well as colon cancer (Hantraye et al., 1996; Simonian and Coyle, 1996; Hooper et al., 1997; Takahashi et al., 1997; Heiss et al., 2001). iNOS-initiated overproduction of nitric oxide contributes to the development of cancer by nitrosative deamination of DNA bases, lipid peroxidation, and DNA strand breaks (Sporn and Roberts, 1986; Heiss et al., 2001). Thus, agents that suppress iNOS overexpression have potential therapeutic value when associated with inflammation and carcinogenic processes.

Proinflammatory cytokines such as interleukin 1β (IL1β) are mediators in the pathogenesis of many chronic inflammatory diseases, including rheumatoid arthritis, a classic example of an autoimmune disorder. IL1β plays a significant role in synovitis and cartilage destruction (Martin and Near, 1995; Krakauer, 2004). Chronic inflammation may be a triggering factor for metabolic syndrome. Stimuli such as overnutrition, physical inactivity, and aging can result in cytokine (like IL1β) hypersecretion, leading to insulin resistance and diabetes in genetically or metabolically predisposed individuals (Esposito and Giugliano, 2004). Therefore, anticytokine therapy in general and the inhibition of IL1β in par-
ticular is a logical clinical target for the treatment of arthritis and prevention of metabolic syndrome and cardiovascular diseases.

Down-regulation of cyclooxygenase 2 (COX2) has been used as another important target for the relief of inflammation (Krakauer, 2004). Elevated expression levels of COX2 leads to excessive production of prostaglandin (PG) via arachidonic acid metabolism (Subbaramaiah et al., 1997; Heiss et al., 2001). In addition to their role as proinflammatory mediators, PGs suppress immune functions, inhibit apoptosis, enhance proliferation, and increase the invasiveness of cancer cells (Goodwin and Coppen, 1983; Ben-Av et al., 1995; Sheng et al., 1998; Heiss et al., 2001). Hence, inhibition of COX2 expression also provides a strategy to treat cancer (Steinbach et al., 2000; Heiss et al., 2001).

Inflammatory diseases are currently treated with steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) (Langman, 1998). Unfortunately, both of these widely prescribed drug classes have significant negative side effects, reducing their use in certain segments of the population (Juni et al., 2005; Pathak et al., 2005). Hence, there is a need to develop new drugs with novel modes of action that do not produce considerable side effects. Natural product-based anti-inflammatory agents with a transcriptional mode of action, good efficacy, and lower risk of side effects offer promising treatment and prevention of inflammation-related conditions. In this report, we summarize the anti-inflammatory activities of two extracts from Barbarea verna seeds rich in PEITC, Barbarea seed preparation (BSP) and PEITC essential oil (PEO). PEO is an enriched version of BSP using an alternative extraction method, creating an extract ideal for use in a mammalian system. Because PEITC is the primary active component of both PEO and BSP, the effect of synthetic PEITC was compared with that of the two Barbarea extracts. We have also compared the activity of PEITC with other forms of isothiocyanates and two commonly used anti-inflammatory drugs to gauge its relative effectiveness as an anti-inflammatory agent. Carrageenan-induced inflammation in the rodent paw presents a classic model of edema formation and hyperalgesia (Krakauer, 2004). This model was used for the in vivo experiments. For all in vitro studies, expression and hyperalgesia (Krakauer, 2004). For all in vitro studies, expression of known proinflammatory marker genes, such as IL1β, iNOS, and COX2, were examined in LPS-stimulated mouse macrophages (RAW 264.7).

Materials and Methods

Chemicals and Biochemicals. PEITC with 99% purity, other (phenyl, tertiary-butyl, allyl, and isopropyl) forms of isothiocyanates, antibiotics, acetyl salicylate (aspirin, C9H8O4), and LPS were purchased from Sigma Chemicals (St. Louis, MO). All other chemicals, including cell culture media, were obtained from Invitrogen Inc. (Carlsbad, CA). Reagents used in quantitative PCR, including enzymes were supplied by Stratagene Inc. (La Jolla, CA). The content of a Vioxx (Merck, Whitehouse Station, NJ) 25-mg capsule dissolved in 95% ethanol alcohol was used as a rofecoxib (C13H12O7S) (stock solution, 10 mg/ml). Winter cress seeds were obtained from AlfniNOS of known proinflammatory marker genes, such as IL1β, iNOS, and COX2, were examined in LPS-stimulated mouse macrophages (RAW 264.7).

Preparation of BSP and PEO. PEO (containing >95% PEITC) was extracted by hydrodistillation of ground winter cress seed for 4 to 5 h with a modified Clevenger apparatus. Generally, 200 g of ground seed plus 1 liter of distilled water would yield 1 to 2 ml of essential oil after hydrodistillation. This process was repeated to produce larger quantities of oil.

For the production of BSP, 200 g of finely ground winter cress seed was soaked with 100 ml of distilled water. The wet material was thinly spread on a disposable aluminum tray (230 × 280 cm) and floated in a covered water bath at 37°C for 20 min followed by lyophilization to a final temperature of 18°C (Ribnicky et al., 2001). To fortify the seed mixture, 22 g of PEO was dissolved in 100 ml of 95% ethanol and added to the dried seed material as described above and mixed until the moisture was evenly distributed. After the seed mixture was evenly spread into the aluminum tray (230 × 280 cm), the ethanol was evaporated in a fume hood for 6 to 8 h. The resulting dried material was defined as BSP, and the PEITC content was measured to be 7.8% by gas chromatography/mass spectrometry (GC/MS).

GC/MS Analysis. For analysis, 1 g of BSP was dissolved into 50 ml of ethyl acetate and particulates were removed by centrifugation at 2000g for 10 min. Samples were injected into a GC/MS Hewlett-Packard mass spectrometer (model 6890N/5973N; Agilent, Palo Alto, CA) equipped with a 30-m × 0.25-mm DB-5MS fused silica capillary column (J&W Scientific, Folsom, CA). Chromatographic parameters were as follows: injection temperature at 150°C, and initial oven temperature at 50°C for 5 min followed by a ramp at 30°C/min to 280°C for 3 min. The MS was operated in the scanning mode from 50 to 650 m/z. The retention time of PEITC was 11.3 min and appeared as the primary compound in the BSP (Ribnicky et al., 2001). The major ion of PEITC has a mass of 91 m/z and molecular ion a mass of 163 m/z. The abundance of these ions and the integration value of the entire peak were used together with standard curves created from a PEITC chemical standard to quantify the PEITC concentration in BSP.

Rat Paw Edema Anti-Inflammatory Assay. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Adult male Wistar rats (100–200 g) were used throughout this study. Fifteen rats, five per cage, were housed in a room maintained at a constant temperature of 24–26°C with 12-h light/dark cycle and had free access to food and water. Before experimentation, animals had 1 week to adapt to the conditions of the facility. Prior to the start of the experiment, body weights were measured individually to determine proper treatment dose, and animals were randomly divided into three different groups of five rats. Initial paw sizes (basal volume) were measured by a volume displacement method using a digital plethysmometer (Ugo Basile, Comerio VA, Italy).

Paw edemas were induced by subcutaneous injection of 100 μl of 1% lambda carrageenan solution (Sigma) (w/v solution in saline, 0.9% NaCl) in the plantar aponeurosis of the right hind paw. Carrageenan is a sulfated polysaccharide [International Union of Pure and Applied Chemistry (IUPAC) nomenclature unavailable] that promotes acute inflammation by activating proinflammatory cells. One hour after carrageenan injection, PEO and aspirin (Sigma) were orally gavaged at 200-mg/kg body weight. An equal volume of the vehicle (4% apricot kernel balm) was given to the control group. Edemas were measured 3, 5, 24, and 48 h after PEO and aspirin treatments. Time-dependent paw edema size reduction reflected the anti-inflammatory effect of the specific treatment. The increase in volume and the percent change (Fig. 1) caused by the irritant were estimated after subtracting the basal volume of the paw before injection. A lower numerical value (in percent) would indicate stronger anti-inflammatory activity.

Macrophone Cell Culture Assay. The mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB-71; obtained from American Type Culture Collection) was maintained in Dulbeco’s modified Eagle’s medium (Invitrogen Inc.) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were kept in a 37°C incubator with 5% CO2.
Cells were subcultured by scraping when plates reached 90% confluence with a 1:5 ratio in fresh medium. Cells were seeded at a density of 0.4 x 10^6 cells per well (viable cell counts were counted out by trypsin blue staining using a hemocytometer) in 24-well plates 12 h prior to treatment. The cells were then treated with test compounds at predetermined doses for 2 h before elicitation with LPS at 1 μg/ml for an additional 6 h. In the case of the time course experiment, to compare the activities of rofecoxib and PEITC (Fig. 5), longer incubations were carried out. For every experiment, one positive control (cells treated only with LPS) and one negative control (cells without any treatment) were included. Two replicates were made for both the treatments and the controls. At the end of the treatment period, cells were harvested in TRIzol reagent (Invitrogen Inc.) for subsequent cellular RNA extraction. DNA was extracted using DNAasy (Invitrogen Inc.) following the manufacturer's protocol. Total RNA was extracted from RAW macrophages using TRIzol reagent (Invitrogen Inc.) following the manufacturer’s instructions. RNA was quantified spectrophotometrically by absorption measurements at 260 and 280 nm using the NanoDrop system (NanoDrop Technologies Inc., Wilmington, DE). The quality of RNA was assessed by separation using gel electrophoresis. RNA was then treated with DNase I (Invitrogen Inc.) following the manufacturer’s guidelines to remove any traces of DNA contamination. The cDNAs were synthesized using 3 μg of RNA for each sample using iScript Reverse Transcriptase, an RNA-dependent DNA polymerase (Stratagene), following the manufacturer’s protocol.

Quantitative Polymerase Chain Reaction and Data Analysis. The synthesized cDNAs were diluted 4-fold. Two microliters of each diluted sample were added to 0.5-μl gene-specific primers (6 μM; oligos synthesized by IDT Inc., Coralville, IA) and 12.5 μl of Brilliant SYBR green PCR master mix (2x) (Stratagene) containing green jump-start Taq ready mix, and final volume was brought to 25 μl by adding sterile distilled water. ROX (Stratagene) was used as an internal dye. To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows (forward and reverse pairs of the primers are indicated as “F” and “R”, respectively): β-actin (accession number NM_007393), F: 5’-AAGCTTGAAGAGTGACCCAGAT-3’, R: 5’-CACAGGCTTGAT-GGTCAGCT-3’; COX2- (accession number NM_011198), F: 5’-TGGTGCTGCTGCTGATGATG-3’, R: 5’-CTGTTAAACGCTGATGGTGTT-G-3’; iNOS- (accession number XM_147149), F: 5’-CCCTCTCTGATC- TTGGTGGTGA-3’, R: 5’-CACCCAGCTCCTGGAA-3’; and IL1β- (accession number NM_008361), F: 5’-CAACAAAGATGATATT- CTCCAGT-3’, R: 5’-GATCCACACTCTCCAGTCGCA-3’.

PCR amplifications were performed on MX3000p system (Stratagene) using one cycle at 50°C for 2 min, one cycle of 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The dissociation curve was completed with one cycle of 1 min at 95°C, 30 s of 55°C, and 30 s of 95°C. Nonreverse transcription control and no template control were included in the PCR program as quality control steps.

RNA expressions for COX2, iNOS, and IL1β, normalized with respect to the expression of housekeeping β-actin gene, were analyzed using the ΔΔCt method (Winer et al., 1999). The ΔΔCt values obtained from these analyses directly reflect the relative mRNA quantities for the specific gene in response to a particular treatment (Figs. 2, 3, 4, and 5). A value of less than 1.0 indicates transcriptional down-regulation (inhibition of gene expression) compared with LPS positive control, which shows maximum genetic induction (1.0). Therefore, lower values indicate greater anti-inflammatory activity. The changes in gene expression as reflected by ΔΔCt values can also be expressed as percentage of genetic inhibition [(1 – ΔΔCt) x 100], indicating anti-inflammatory properties of the test compounds. Values higher than 1.0 imply overexpression of the particular gene in excess of LPS stimulation. Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. All samples were run in duplicate.

Statistical Analysis. Experimental observations (Figs. 1–6) are expressed as the mean ± S.E. In Fig. 1, the significance of any treatment (PEO or aspirin) over the untreated control was determined by Student’s t test as described by Press et al. (1992). The t test was used in this case because the number of treatments was less than 3. The t test was also performed for Fig. 5 for the same reason. For Figs. 2 to 4 and 6, one-way ANOVA (analysis of variance) was used to determine the significance of treatments. To determine the specificity of the treatments, post-ANOVA Tukey’s Honestly Significant Difference multiple means comparison test (Tukey, 1972) was carried out to determine the significance of difference between control and treatments. Treatments were considered significantly different if p < 0.01 for gene expression data and p < 0.05 for the animal data.

Results

In the rat paw edema experiment, inflammation was induced by carrageenan injection for 1 h and then treated with PEO, aspirin, or vehicle (see Materials and Methods). The effect of PEO in reducing acute inflammation was found to be similar to a comparable dose of aspirin, a known NSAID (Fig. 1). Three hours after the treatment with PEO, inflammation was significantly reduced compared to the control group. However, aspirin showed a more pronounced anti-inflammatory effect with a larger reduction in paw volume compared to the control and PEO-treated groups. The results also indicated that PEITC, a compound synthesized from PEO, showed potential anti-inflammatory activity, as evidenced by the reduced paw edema in the treated group compared to the control. Further experiments and studies are needed to confirm these findings and to understand the mechanisms involved in the anti-inflammatory effects of PEO and PEITC.
was reduced by 50% compared with the untreated group \((p < 0.001)\). These data indicate that PEO was quickly absorbed and biologically available. After 5 \((p < 0.001)\) and 24 \((p < 0.05)\) h, inflamed paw sizes continued to shrink, showing that the treatment was pharmacologically active for a prolonged time. However, with the further incubation up to 48 h, the statistical significance between the effects in treated and untreated group was lost \((p > 0.05)\) (Fig. 1).

The in vitro experiments were designed to quantify the relative amount of transcripts for target genes \((COX2, iNOS, \text{and } IL1\beta)\) within the total RNA in individual cell batches undergoing dose-dependent treatments with synthetic PEITC (Fig. 2A), BSP (Fig. 2B), aspirin (Fig. 3), rofecoxib (Fig. 4), and four other forms of isothiocyanates (phenyl, tertiary-butyl, allyl, and isopropyl) (Fig. 6). All experiments were started with an equal number of cells for each treatment. For each assay, two control sets were monitored (Figs. 2, 3, and 4). The positive control (treated only with LPS) showed the maximum up-regulation of the marker genes (except in the case of COX2, which is discussed in the following section). The negative control (received no LPS treatment) maintained a constant amount of transcripts for all constitutively expressed genes and served as a reference baseline. Since noncytotoxic doses were predetermined by the MTT assay (data not shown), the observed changes in gene expression (genetic down-regulation) were not due to cell death. A change (decrease) in relative mRNA quantity, compared with the positive control, indicated the inhibitory effect and hence, anti-inflammatory activity of the particular...
treatment. Monitoring the expression of \( /H_9252 \)-actin, a constitutively expressed housekeeping gene, served as a quality control step for determining RNA degradation during the course of the assay.

The in vitro experiments showed synthetic PEITC and BSP to be highly anti-inflammatory (significant at \( p < 0.001 \) by ANOVA) in assays with all of the target genes—\( /H_9252 \), \( /H_9252 \), \( /H_9252 \), and \( /H_9252 \) (Fig. 2). Above 20 \( \mu M \) PEITC and 50 \( \mu g/ml \) BSP (equivalent to 23.5 \( \mu M \) PEITC content), the inhibitory effects measured by gene down-regulation were almost 100% (corresponding to a relative mRNA quantity of ~0.00) (Fig. 2). One to 40 \( \mu M \) PEITC (Fig. 2A) and 1.56 to 100 \( \mu g/ml \) BSP (Fig. 2B) were tested to determine the dose response. Pronounced dose-dependent inhibition of \( /H_9252 \) and \( /H_9252 \) further confirmed the activity of PEITC and BSP. For \( /H_9252 \), the inhibitory effects of both PEITC and BSP were not clearly dose-dependent but were strong only above a certain concentration (≥20 \( \mu M \)) (Fig. 2). Either complete inhibition (≥20 \( \mu M \)) or minimal suppression (<20 \( \mu M \)) of \( /H_9252 \) was observed. Furthermore, low concentrations of PEITC (~1 \( \mu M \)) reproducibly induced the levels of \( /H_9252 \) gene expression to the levels above those observed with LPS alone (Fig. 2). However, in the absence of LPS, induction of \( /H_9252 \) expression was not achieved in RAW macrophages by either PEITC or BSP (data not shown). Nevertheless, in all experiments, PEITC and BSP showed very similar responses (statistical significance in both cases were at \( p < 0.001 \) by ANOVA),
further confirming that PEITC is the primary active compound in BSP.

The effects of aspirin (Fig. 3) and rofecoxib (Figs. 4 and 5) on the mRNA levels of the inflammation-related genes were much less than that of PEITC. Aspirin showed minor dose-dependent inhibition of iNOS, whereas rofecoxib showed some dose-dependent inhibition of COX2 expression. Rofecoxib also displayed a time-dependent activity for both COX2 and iNOS (Fig. 5, A and B). When activity of PEITC was compared with the other forms of isothiocyanates (Fig. 6), PEITC was by far the most inhibitory in both iNOS and IL1β assays, indicating highest anti-inflammatory activity. Only allyl isothiocyanate showed somewhat comparable inhibitory effects on iNOS and IL1β expression to that of PEITC at 5 μM but had higher cytotoxicity than PEITC in the MTT assay at 10 μM. All forms of isothiocyanate including PEITC showed persistent high mRNA-relative quantity in the COX2 assay (Fig. 6A).

Discussion

*Barbarea verna* seeds are a rich source of PEITC with a potential for providing natural protection from environmental and dietary toxins (Ribnicky et al., 2001). Two PEITC-containing preparations were made from *B. verna* seeds, an essential oil containing >95% PEITC (PEO) and a ground seed extract enriched with PEO, containing 7.8% PEITC (BSP). PEO was used instead of BSP in animal experiments because of the difficulty of gavaging animals with BSP, which is a granular and insoluble powder. BSP and PEITC standards were used for all other experiments.

Here, we present the first preclinical study of a botanical formulation containing PEITC that shows pronounced anti-inflammatory activity in vivo (Fig. 1). The rat paw edema model was chosen to study acute inflammation in a mammalian system. PEO was bioavailable when orally gavaged in rats and had pharmacological activity as soon as 3 h after carrageenan injection. No obvious loss in growth or appetite occurred in any group. PEO demonstrated similar efficacy to that of aspirin in vivo, significantly reducing induced paw edema for up to 24 h. These data indicate PEO may be an excellent candidate for development as a therapy for inflammation.

In vitro, our data demonstrate that PEITC and BSP were able to inhibit the LPS-elicited induction of IL1β (Fig. 2), a positive marker for inflammation, metabolic syndrome, and immune suppression. In addition, PEITC and BSP were able to inhibit the LPS-elicited transcription of inducible proinflammatory enzyme coding genes like COX2 and iNOS. All three genes have a binding site in their promoter region for nuclear factor kappa B (NFκB) (Baeuerle and Baltimore, 1996; Lee et al., 2003; Yamamoto and Gaynor, 2004). In vitro effects of PEITC on the inhibition of NFκB-mediated inducible nitric oxide production and PG synthesis have been previously reported in context of its chemopreventive activity (Heiss et al., 2001; Chen et al., 2003; Gerhauser et al., 2003; Rose et al., 2005). Therefore, it is likely that transcriptional down-regulation of inflammatory genes by PEITC and BSP was mediated through the transcription factor NFκB.

At low concentrations, PEITC and BSP up-regulated the COX2 gene compared with LPS control (Fig. 2). The experiment was repeated several times with the same results; however, when cells were treated in the absence of LPS, COX2 expression was unaffected by either PEITC or BSP. Probable explanations for such effects may lie in the complex, pleiotropic activities of numerous interplaying cellular factors, such as feedback mechanisms of genetic regulation.

![Fig. 6. Effects of PEITC and other isothiocyanates (phenyl isothiocyanate, tertiary-butyl isothiocyanate, isopropyl isothiocyanate, and allyl isothiocyanate) on the expression of the inflammation-related genes in the LPS-stimulated RAW264.7 macrophages (three replicates for each treatment at 5 and 10 μM). Changes in gene expression were measured by comparing mRNA quantity relative to LPS positive control that is normalized to a value of 1.00. Lower values represent greater inhibitory effects. Values are mean ± S.E., p < 0.05; **, p < 0.01; ***, p < 0.001 (post-ANOVA comparison with LPS-treated positive control); data were not obtained for allyl isothiocyanate at 10 μM because of cytotoxicity; COX2 assay (A), iNOS assay (B), and IL1β assay (C).](jspetjournals.org/file/331.png)
within a cell (Krakauer, 2004). It is also possible that in addition to NFκB, other transcription controlling factors may be involved in partly controlling the COX2 expression (von Knethen and Brune, 2000).

When we compared the effects of aspirin with those of BSP and synthetic PEITC on the transcription of iNOS, COX2, and IL1β, comparable inhibitions were not observed at the concentrations tested (Fig. 3). This was not surprising, however, because existing data (Kwon et al., 1997) showed that aspirin inhibited iNOS at the post-transcriptional level. Therefore, it is likely that PEITC in the form of BSP or PEO possesses a mechanism of anti-inflammatory action that is different from that of aspirin.

The safety of selective COX2 inhibitor drugs has recently become controversial (Junii et al., 2005). Because PEITC and BSP inhibit COX2 expression (≥20 μM), an in vitro comparison of actions between rofecoxib and BSP/PEITC (Figs. 4 and 5) was carried out. Rofecoxib (10 μM) was previously shown to suppress COX2 and iNOS expression levels after 18 h of LPS activation, and the inhibition was LPS-dose-dependent (effect was observed only at 100 ng/ml or lower LPS concentrations) (Callejas et al., 2003). In our experimental system optimized with 6 h of LPS activation at 1 μg/ml, rofecoxib was unable to significantly inhibit gene expression (Fig. 4). Therefore, longer incubations (9 and 21 h) (Fig. 5) were investigated using the standard (1 μg/ml) and 10-fold lower (100 ng/ml) LPS concentrations (data not shown). Because BSP showed very similar activities to that of synthetic PEITC in the prior experiments, comparison of responses in response to variation in time and LPS concentrations were limited to rofecoxib and synthetic PEITC only (Fig. 5). The following observations were noted when synthetic PEITC (5 μM) was compared with 5 μM rofecoxib: 1) PEITC suppresses the expression of proinflammatory genes (IL1β, iNOS, and COX2) with LPS activation at 1 μg/ml. In contrast, rofecoxib action of similar magnitude is only achievable on COX2 and iNOS with at least a 10-fold lower (100 ng/ml) LPS activation (data not shown). 2) The effect of PEITC was most pronounced on IL1β, a known marker for metabolic syndrome, inflammation, and immune suppression (Figs. 2 and 4C). Rofecoxib did not show any effect on this proinflammatory cytokine. Longer incubation did not show any change in activity of PEITC or rofecoxib on IL1β expression (Fig. 5C). 3) Both PEITC and rofecoxib showed suppression of iNOS expression; PEITC was the stronger inhibitor. Changes in incubation time had similar effects for both, showing an increase in suppression up to the 9th h, followed by a decline at the 21st h (Figs. 4B and 5B). 4) Rofecoxib showed very strong activity on COX2 inhibition at the 21st h. The activity of rofecoxib increased steadily with the duration of treatment. The activity of PEITC on COX2 suppression also increased significantly but only up to the 9th h (Fig. 5A). These observations indicate that the anti-inflammatory mechanisms of actions of PEITC and hence, that of BSP, are likely to be distinct from that of rofecoxib.

In summary, PEITC, applied as a synthetic compound or as a botanical formulation, is capable of suppressing the expression of the inflammation-related genes in the in vitro assays. The rat study shows that PEO is biologically available and pharmacologically active, reducing acute inflammation in a mammalian experimental system. No obvious loss in growth or appetite occurred in any group. The safety of BSP and PEO is further substantiated by the fact that winter cress has been consumed by people for centuries, apparently without any known adverse effects. In addition, a previous report (Hecht, 1997) on hematology and blood chemistry of prolonged PEITC-treated rats did not show any toxic effects. Nevertheless, detailed preclinical pharmacology and toxicology studies with BSP are needed to further establish BSP as a promising candidate for human clinical use.

Acknowledgments

We thank Dr. Christophe Ripoll for sharing information on anti-inflammatory gene expression assays, which greatly expedited standardization of in vitro techniques. We also thank Reneta Poulева and Irina Aranovich for excellent technical assistance and Dr. Barbara Schmidt for critical reading of the manuscript.

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