In vitro and *in vivo* anti-inflammatory activity of a seed preparation containing phenethylisothiocyanate

Moul Dey, David Ribnicky, Anvar G. Kurmukov, and Ilya Raskin

Rutgers University, Biotech Center, 59 Dudley Road, New Brunswick, NJ 08901, USA

a) **Anti-inflammatory activity of PEITC**

b) Corresponding author:

Prof. Ilya Raskin

Rutgers University, Biotech Center, 59 Dudley Road, New Brunswick, NJ 08901

Tel.: +1-732-932-8165; Fax: +1-732-932-6535

Email : raskin@aesop.rutgers.edu

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BSP: *Barbarea verna* seed preparation, COX: cyclooxygenase, GC/MS: gas chromatography/mass spectrometry, IL1β: interleukin1β, iNOS: inducible nitric oxide synthase, LPS: lipopolysaccharide, NFκB: nuclear factor kappaB, NSAID: non-steroidal anti-inflammatory drug, PEITC: phenethylisothiocyanate, PEO: PEITC essential oil, PG: prostaglandin, RT-PCR: reverse transcription polymerase chain reaction, SE: standard error

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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 to that of the non-steroidal anti-inflammatory drug (NSAID), aspirin. The seed preparation, in a concentration-dependant manner, reduced the mRNA levels of inflammation-related genes such as, the inducible forms of cyclooxygenase (COX2), nitric oxide synthase (iNOS) and the pro-inflammatory cytokine, interleukin (IL1β) in lipopolysaccharide (LPS)-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 out of five different forms of isothiocyanate tested for their effects on *in vitro* pro-inflammatory gene expression. *In vitro* activity of the seed preparation was also compared to 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 transcriptions of pro-inflammatory genes.

INTRODUCTION

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 chemo-preventive properties. Also other related health-promoting effects in context of its anti-carcinogenic activities, including anti-inflammatory, have been reported (Rose et al., 2005; Chen et al., 2003; Gerhauser et al., 2003; Heiss et al., 2001; Stoewsand, 1995).

Chronic inflammation, carcinogenesis, autoimmune disorders and metabolic syndrome are mechanistically linked (Esposito and Giugliano, 2004; Heiss et al., 2001; Ohshima and Bartsch, 1994) 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, pro-inflammatory enzyme, iNOS is involved in multiple sclerosis, Parkinson's and Alzheimer's diseases, as well as colon cancer (Heiss et al., 2001; Hooper et al., 1997; Simonian and Coyle 1996; Takahashi et al., 1997; Hantraye et al., 1996). iNOS-initiated over-production of NO contributes to the development of cancer by nitrosative deamination of DNA bases, lipid peroxidation and DNA strand breaks (Heiss et al., 2001; Sporn and Roberts, 1986). Thus, agents that suppress iNOS over-expression have potential therapeutic value when associated with inflammation and carcinogenic processes.

Pro-inflammatory cytokines, such as 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 (Krakauer, 2004; Martin and Near 1995). Chronic inflammation may be a triggering factor for metabolic syndrome. Stimuli such as over nutrition, physical inactivity and aging can result in cytokine (like IL1β) hyper-secretion, leading to insulin resistance and diabetes in genetically or metabolically predisposed individuals (Esposito and Giugliano, 2004). Therefore, anti-cytokine therapy in general and the inhibition of IL1β in particular is a logical clinical target for the treatment of arthritis and prevention of metabolic syndrome and cardiovascular diseases.

Down-regulation of 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 (Heiss et al., 2001; Subbaramaiah et al., 1997). In addition to their role as pro-inflammatory mediators, PGs suppress immune functions, inhibit apoptosis, enhance proliferation, and increase the invasiveness of cancer cells (Heiss et al., 2001; Sheng et al., 1998; Ben-Av et al., 1995; Goodwin and Ceuppens, 1983). Hence, inhibition of COX2 expression also provides a strategy to treat cancer (Heiss et al., 2001; Steinbach et al., 2000).

Inflammatory diseases are currently treated with steroidal and non-steroidal 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. Since 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 classical model of edema formation and hyperalgesia (Krakauer, 2004). This model was used for the *in vivo* experiments. For all *in vitro* studies, expression of known pro-inflammatory marker genes, such as IL1β, iNOS and COX2 were examined in LPS-stimulated mouse macrophages (RAW 264.7).

METHODS

Chemicals and biochemicals

PEITC with 99% purity, other (phenyl, tertiary-butyl, allyl, isopropyl) forms of isothiocyanates, antibiotics, acetyl salicylate (aspirin, $C_9H_8O_4$) and LPS were purchased from Sigma Chemicals (St. Louis, MO). All other chemicals, including cell culture media were obtained from Invitrogen Inc., (Carslad, CA). Reagents used in quantitative PCR, including enzymes were supplied by Stratagene Inc., (LaJolla, CA). The content of a Vioxx 25 mg capsule dissolved in 95% ethyl alcohol was used as a rofecoxib ($C_{17}H_{14}O_4S$) (stock solution 10 mg/ml). Winter cress seeds were obtained from Alf Christiansen Seed Co., Mount Vernon, WA. RAW 264.7 cell line (ATCC TIB-71) was provided by American Type Culture Collection, VA.

Preparation of BSP and PEO

PEITC essential oil (PEO; containing >95% PEITC) was extracted by hydrodistillation of ground winter cress seed for 4-5 h with a modified Clevenger apparatus. Generally, 200 g of ground seed plus 1 L of distilled water would yield 1-2 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 cm X 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). In order 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 cm X 280 cm), the ethanol was evaporated in a fume hood for 6-8 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 2000 x g for 10 min. Samples were injected into a GC/MS (model 5890/5971, Hewlett-Packard mass spectrometer equipped with a 30-m X 0.25 mm DB-5MS fused silica capillary column (J&W Scientific, Folsom CA). Chromatographic parameters were as follows: injection temperature at 150°C, 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 of mass 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 Institute of Health. Adult male Wistar rats (100-200g) were used throughout this study. Fifteen rats, 5 per cage, were housed in a room maintained at a constant temperature of 24-26ºC with 12-h light/dark cycle and had free access to food and water. Before experimentation, animals had one 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 5 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 Inc., USA) (w/v solution in saline, 0.9% NaCl) in the plantar aponeurosis of the right hind paw. Carrageenan is a sulfated polysaccharide (IUPAC nomenclature unavailable) that promotes acute inflammation by activating pro-inflammatory cells. One h after carrageenan injection, PEO and aspirin (Sigma Inc., USA) 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 percentage change (Figure 1) caused by the irritant was estimated after subtracting the basal volume of the paw before injection. A lower numerical value (in %) would indicate stronger anti-inflammatory activity.

Macrophage cell culture assay

The mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB-71 obtained from American Type Culture Collection, VA 20108, USA) was maintained in Dulbecco's Modified Eagle Medium (DMEM, 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 ºC incubator with 5% CO_2. Cells were sub-cultured by scraping when plates reached 90% confluence with a 1:5 ratio in fresh medium.

Cells were seeded at a density of $0.4x10^6$ cells per well (viable cell counts were carried out by trypan blue staining using a hemocytometer) in 24 well plates 12 h prior to treatment. The cells were then treated with test compounds at pre-determined doses for 2 h before elicitation with LPS at 1 µg/ml for an additional 6 h. In case of the time-course experiment to compare the activities of rofecoxib and PEITC (Figure 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. BSP stock solution was prepared by dissolving BSP (50 mg/ml) in 95% ethyl alcohol for 15 min by sonication. Sonication released PEITC into the clear supernatant which was later used for cell culture treatment.

Cell viability assay and dose range determination

Cell viability was measured by the MTT assay (Mosmann, 1983). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) (100 µg/ml, Sigma, St. Louis, MO) was added to the medium in each well and plates were incubated for 3 h at 37 ºC. Medium was then removed and dimethyl sulfoxide (200 µL) was added to each well to solubilize the purple formazan crystals created by mitochondrial dehydrogenase reduction of MTT. After 5 min of additional incubation, absorbance was read at 550 nm on a micro plate spectrophotometer (Molecular Devices, Sunnyvale, CA). The data were expressed as percent cell viability compared to control (dimethyl sulfoxide, 0.1%) (data not shown). The concentrations of test reagents that showed significant ($p<0.001$) cell viability were further selected for *in vitro* gene expression assays.

Total RNA extraction, purification and cDNA synthesis

Total RNA was extracted from RAW macrophages using Trizol reagent (Invitrogen Inc.) following the manufacturer instructions. RNA was quantified spectrophotometrically by absorption measurements at 260nm and 280nm using the NanoDrop system (NanoDrop Technologies Inc. DE, USA). Quality of RNA was assessed by separation using gel-electrophoresis. RNA was then treated with DnaseI (Invitrogen Inc.) following the manufacturer guidelines, to remove any traces of DNA contamination. The cDNAs were synthesized using 3µg of RNA for each sample using Stratascript Reverse Transcriptase, an RNA dependent DNA polymerase (Stratagene, La Jolla, CA), following the manufacturer protocol.

Quantitative polymerase chain reaction (qPCR) and data analysis

The synthesized cDNAs were diluted 4-fold. Two microliters of each diluted sample were added to 0.5µl gene specific primers (6µM, oligos synthesized by IDT Inc. USA), 12.5µl of Brilliant SYBR green PCR master mix (2X) (Stratagene, La Jolla, CA) containing green jump-start Taq ready mix and final volume was brought to 25 µL by adding sterile distilled water. ROX (Stratagene, La Jolla, CA) was used as an internal dye. To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using the Primer Express® vers. 2.0 software (Applied Biosystem, Foster City, CA) as follows:

β-actin (accession number NM_007393);	F : 5'-AACCGTGAAAAGATGACCCAGAT-3'
	R: 5'-CACAGCCTGGATGGCTACGT-3'
COX2- (accession number NM_011198);	F: 5'-TGGTGCCTGGTCTGATGATG-3'
	R: 5'-GTGGTAACCGCTCAGGTGTTG-3'
iNOS- (accession number XM_147149);	F: 5'-CCCTCCTGATCTTGTGTTGGA-3'

R: 5'-TCAACCCGAGCTCCTGGAA-3'

IL1β- (accession number NM_008361); F: 5'-CAACCAACAAGTGATATTCTCCATG-3'

R: 5'-GATCCACACTCTCCAGCTGCA-3'

Forward and reverse pairs of the primers are indicated above as "F" and "R", respectively. PCR amplifications were performed on MX3000p system (Stratagene, La Jolla, CA) using 1 cycle at 50 ºC for 2 min, 1 cycle of 95 ºC for 10 min, followed by 40 cycles of 15seconds at 95 ºC and 1 min at 60 ºC. The dissociation curve was completed with one cycle of 1 minute at 95 ºC, 30 seconds of 55 ºC and 30 seconds of 95 ºC. NRT (non-RT control) and NTC (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 (Figures 2, 3, 4, 5). A value less than 1.0 indicates transcriptional down-regulation (inhibition of gene expression) as compared to 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 genetic inhibition [(1- ΔΔCt) x100] indicating anti-inflammatory properties of the test compounds. Values higher than 1.0 imply over-expression 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 (Figures 1-6) are expressed as the mean ± SE. In Figure 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 as the number of treatments was less than 3. The t-test was also performed for Figure 5 due to the same reason. For Figures 2-4 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 HSD multiple mean 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 one h, and then treated with PEO, aspirin or vehicle (see method). The effect of PEO in reducing acute inflammation was found to be similar to a comparable dose of aspirin, a known NSAID (Figure 1). Three h after the treatment with PEO, inflammation was reduced by 50% as compared to the untreated group ($p<0.001$). These data indicate that PEO was quickly absorbed and biologically available. After 5 ($p<0.001$) and 24 ($p\leq0.05$) 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 ($p\geq0.05$) (Figure 1).

The *in vitro* experiments were designed to quantify the relative amount of transcripts for target genes (COX2, iNOS and IL1β) within the total RNA in individual cell batches undergoing dose-dependent treatments with synthetic PEITC (Figure 2A), BSP (Figure 2B), aspirin (Figure 3), rofecoxib (Figure 4) and four other forms of isothiocyanates (phenyl, tertiary-butyl, allyl, isopropyl) (Figure 6). All experiments were started with an equal number of cells for each treatment. For each assay, two control sets were monitored (Figures 2, 3, 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. As non-cytotoxic doses were pre-determined 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 to the positive

control, indicated the inhibitory effect and hence anti-inflammatory activity of the particular treatment. Monitoring the expression of β-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- COX2, iNOS and IL1β (Figure 2). Above 20 μM PEITC and 50 μg/ml BSP (equivalent to 23.5 μM PEITC content), the inhibitory effects measured by gene down regulation were almost 100% (corresponding to a relative mRNA quantity of ~0.00) (Figure 2). One to 40 μM PEITC (Figure 2A) and 1.56 to 100 μg/ml BSP (Figure 2B) were tested to determine the dose response. Pronounced dose-dependent inhibition of iNOS and IL1β further confirmed the activity of PEITC and BSP. For COX2, the inhibitory effects of both PEITC and BSP were not clearly dose dependent but were strong only above a certain concentration (≥20 μM) (Figure 2). Either complete inhibition (≥20 μM) or minimal suppression (<20 μM) of COX2 was observed. Furthermore, low concentrations of PEITC (~ 1 μM) reproducibly induced the levels of COX2 gene expression to the levels above those observed with LPS alone (Figure 2). However, in absence of LPS, induction of COX2 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 (Figure 3) and rofecoxib (Figures 4, 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, while rofecoxib showed some dose-dependent inhibition of COX2 expression. Rofecoxib also displayed a time-dependent activity for both COX2 and iNOS

(Figure 5A, 5B). When activity of PEITC was compared to the other forms of isothiocyanates (Figure 6), PEITC was by far the most inhibitory in both iNOS and IL1β assays, indicating highest anti-inflammatory activity. Only ally-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 (Figure 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 difficulty gavaging animals with BSP, which is a granular and insoluble powder. BSP and PEITC standard were used for all other experiments.

Here we present the first pre-clinical study of a botanical formulation containing PEITC that shows pronounced anti-inflammatory activity *in vivo* (Figure 1). The rat paw edema model was chosen to study acute inflammation in a mammalian system. PEO was bio-available 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 (Figure 2), a positive marker for inflammation, metabolic syndrome and immune suppression. Additionally, PEITC and BSP were able to inhibit the LPS elicited transcription of inducible pro-inflammatory enzyme coding genes like COX2 and iNOS. All of these three genes have a binding site in their promoter region for Nuclear Factor kappa B (NFκB) (Yamamoto and Gaynor, 2004; Lee et al., 2003; Baeurerle and Baltimore, 1996). *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 chemo-preventive activity (Rose et al., 2005; Chen et al., 2003; Gerhauser et al., 2003; Heiss et al., 2001). Therefore, it is likely 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 to LPS control (Figure 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 inter-playing cellular factors such as feed-back mechanisms of genetic regulation 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 (Figure 3). This was not surprising, however, since 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 possess a mechanism of anti-inflammatory action that is different from that of aspirin.

The safety of selective COX2 inhibitor drugs has recently become controversial (Juni et al., 2005). Since PEITC and BSP inhibit COX2 expression [≥20 μM], an *in vitro* comparison of actions between rofecoxib and BSP/PEITC (Figures 4, 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 concentration) (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 (Figure 4). Therefore, longer incubations (9 and 21 h) (Figure 5) were investigated using the standard (1 μg/ml) and ten fold lower (100ng/ml) LPS concentrations (data not shown). Since BSP showed very similar activities to that of synthetic PEITC in the prior experiments, comparison of activities in response to variation in time and LPS concentrations were limited to rofecoxib and synthetic PEITC only (Figure 5). The following observations were noted when synthetic PEITC [5 μM] was compared to rofecoxib [5 μM]:

I) PEITC suppresses the expression of pro-inflammatory 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 ten fold lower (100 ng/ml) LPS activation (data not shown).

II) The effect of PEITC was most pronounced on IL1β, a known marker for metabolic syndrome, inflammation and immune suppression (Figures 2, 4C). Rofecoxib did not show any effect on this pro-inflammatory cytokine. Longer incubation did not show any change in activity of PEITC or rofecoxib on IL1β expression (Figure 5C).

III) 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 9^{th} h, followed by a decline at the 21^{st} h (Figures 4B, 5B).

IV) Rofecoxib showed very strong activity on COX2 inhibition at the 21^{st} 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 (Figure 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 pre-clinical pharmacology and toxicology studies with BSP are needed to further establish BSP as a promising candidate for human clinical use.

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REFERENCES

Baeuerle PA and Baltimore D (1996) NF-kappa B: ten years after. *Cell* 87: 13-20.

Ben-Av P, Crofford LJ, Wilder RL and Hla T (1995) Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett* 372: 83-87.

Callejas NA, Fernández-Martínez A, Castrillo A, Boscá L and Martín-Sanz P (2003) Selective Inhibitors of Cyclooxygenase-2 Delay the Activation of Nuclear Factor κB and Attenuate the Expression of Inflammatory Genes in Murine Macrophages Treated with Lipopolysaccharide. *Mol Pharmacol* 63: 671-677.

Chen H, Dail HJ and Chang HP (2003) Suppression of Inducible Nitric Oxide Production by Indole and Isothiocyanate Derivatives from Brassica Plants in Stimulated Macrophages. *Planta Med* 69: 696-700.

Esposito K and Giugliano D (2004) The metabolic syndrome and inflammation: association or causation? *Nutr Metab Cardiovasc Dis* 14: 228-232.

Gerhauser C, Klimo K, Heiss E, Neumann I, Gamal-Eldeen A, Knauft J, Liu GY, Sitthimonchai S and Frank N (2003) Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutat Res* 523-524: 163-172.

Goodwin JS and Ceuppens J (1983) Regulation of the immune response by prostaglandins. *J Clin Immunol* 3: 295-314.

Hantraye P, Brouillet E, Ferrante R, Palfi S, Dolan R, Matthews RT and Beal MF (1996) Inhibition of neuronal nitric oxide synthase prevents MPTP-induced parkinsonism in baboons. *Nat Med* 2: 1017-1021.

Hecht SS (1997) Tobacco and cancer: approaches using carcinogen biomarkers and chemoprevention. *Ann N Y Acad Sci*. 833: 91-111.

Heiss E, Herhaus C, Klimo K, Bartsch H and Gerhäuser C (2001) Nuclear Factor kappa B Is a Molecular Target for Sulforaphane-mediated Anti-inflammatory Mechanisms. *J Biol Chem* 276: 32008-32015.

Hooper DC, Bagasra O, Marini JC, Zborek A, Ohnishi ST, Kean R, Champion JM, Sarker AB, Bobroski L, Farber JL, Akaike T, Maeda H and Koprowski H (1997) Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: implications for the treatment of multiple sclerosis. *Proc Natl Acad Sci USA* 94: 2328-2333.

Krakauer T (2004) Molecular therapeutic targets in inflammation: cyclooxygenase and NFκβ. *Curr Drug Targets* 3: 317-324.

Kwon G, Hill JR, Corbett JA and McDaniel ML (1997) Effects of aspirin on nitric oxide formation and de novo protein synthesis by RINm5F cells and rat islets. *Mol Pharmacol* 52: 398-405.

Langman MJS (1998) Ulcer complications and NSAIDs. *Am J Med* (suppl) 84: 15-19.

Lee AK, Sung SH, Kim YC and Kim SG(2003) Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-alpha and COX-2 expression by sauchinone effects on I-kappaBalpha phosphorylation, C/EBP and AP-1 activation. *Br J Pharmacol* 139: 11-20.

Juni P, Reichenbach S and Egger M (2005) COX2 inhibitors, traditional NSAIDs and the heart. *Brit Med J* 330: 1342-1343.

Martin D and Near SL (1995) Protective effect of the interleukin-1 receptor antagonist (IL-1ra) on experimental allergic encephalomyelitis in rats. *J Neuroimmunol* 61: 241-246.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63.

Ohshima H and Bartsch H (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 305: 253-264.

Pathak SK, Sharma RA, Steward WP, Mellon JK, Griffiths TR and Gescher AJ (2005) Oxidative stress and cyclooxygenase activity in prostate carcinogenesis: targets for chemopreventive strategies. *Eur J Cancer* 41: 61-70.

Press WH, Teukolsky SA, Vetterling WT and Flannery BP (1992) *Numerical recipes, 2nd ed*. Cambridge University Press, Cambridge, UK.

Ribnicky DM, Poulev A, Henry E and Raskin I (2001) Seed of *Barbarea verna* as a rich source of phenethyl isothiocyanate to provide natural protection from environmental and dietary toxins. *J Nutraceuticals, Functional and Med Foods* 3: 43-65.

Rose P, Won YK, Ong CN and Whiteman M (2005) [beta]-Phenylethyl and 8-methylsulphinyloctyl isothiocyanates, constituents of watercress, suppress LPS induced production of nitric oxide and prostaglandin E2 in RAW 264.7 macrophages. *Nitric Oxide* 12: 237-243.

Sheng H, Shao J, Morrow JD, Beauchamp RD and DuBois RN (1998) Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res* 58: 362-366.

Simonian NA and Coyle JT (1996) Oxidative stress in neurodegenerative diseases. *Annu Rev Pharmacol Toxicol* 36: 83-106.

Sporn MB and Roberts AB (1986) Peptide growth factors and inflammation, tissue repair and cancer. *J Clin Invest* 78: 329-332.

Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E and Gordon GB (2000) The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med* 342: 1946-1952.

Stoewsand GS (1995) Bioactive organosulfur phytochemicals in Brassica oleracea vegetables - a review. *Food Chem Toxicol* 33: 537-543.

Subbaramaiah K, Zakim D, Weksler BB and Dannenberg AJ (1997) Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proc Soc Exp Biol Med* 216: 201-210.

Takahashi M, Fukuda K, Ohata T, Sugimura T and Wakabayashi K (1997) Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. *Cancer Res* 57: 1233-1237.

Tukey JW (1972) *Robust Estimates of Location: Survey and Advances*: Princeton Univ. Press, Princeton, USA.

von Knethen A and Brune B (2000) Superinduction of cyclooxygenase-2 by NO and agonist challenge involves transcriptional regulation mediated by AP-1 activation. *Biochemistry* 39: 1532-1540.

Winer J, Jung CK, Shackel I and Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* 270: 41-49.

Yamamoto Y and Gaynor RB (2004) IkappaB kinases: key regulators of the NF-kappaB pathway. *Trends Biochem Sci* 29: 72-79.

FOOTNOTES

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b) Reprints may be sent to

Prof. Ilya Raskin;

Rutgers University, Biotech Center, 59 Dudley Road, New Brunswick, NJ 08901

Email: raskin@aesop.rutgers.edu

c) Institute of Cardiology, Ministry of Health of Republic Uzbekistan, Murtazaeva Street 4, Tashkent 700052, Republic Uzbekistan (AGK)

FIGURE LEGENDS

Figure 1. Anti-inflammatory effect of orally administered PEO (from BSP) and aspirin. Edema induced by subcutaneous injection of 100 µl of 1% carrageenan, 1 h prior to treatments. Increase in paw-edema volumes (ml) were measured at indicated times and the percentage increase was calculated comparing with basal paw volume measured before the experiment. Values are mean± S.E. *, sample size= 5, df= 8, $\boldsymbol{p} \leq 0.05$; ***, $\boldsymbol{p} < 0.001$; significance of any treatment was determined with respect to untreated control.

Figure 2. Pro-inflammatory gene expressions in LPS-activated RAW macrophages. Effect of any treatment (4 replicates) on a specific gene expression was measured by the mRNA quantity relative to the response to LPS activation only (positive control) that is normalized to a value of 1.00; Lower values represent greater inhibitory effects with 0.00 corresponding to a complete inhibition of the induced gene expression; Values are mean ± S.E. *, $\boldsymbol{p} < 0.05$; **, $\boldsymbol{p} < 0.01$; ***$\boldsymbol{p} < 0.001$ (post ANOVA comparison with LPS-treated positive control) (A) Effect of PEITC on mRNA levels of COX2, iNOS and IL1β (B) Effect of BSP on mRNA levels of COX2, iNOS and IL1β; Approximate concentrations of PEITC in BSP are indicated in parenthesis.

Figure 3. Comparative effects of PEITC, BSP and aspirin (4 replicates for each treatment) on gene expressions in LPS-activated RAW macrophages. Concentrations of BSP are in terms of its approximate PEITC content; Effect of any treatment on a specific gene expression was measured by the mRNA quantity relative to the response to LPS activation only (positive control) that is normalized to a value of 1.00; Lower values represent greater inhibitory effects with 0.00

corresponding to a complete inhibition of the induced gene expression; Values are mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$ ((post ANOVA comparison with LPS-treated positive control) (A) COX2 assay, (B) iNOS assay (C) IL1β assay

Figure 4. Comparison between PEITC, BSP and rofecoxib treatments (4 replicates for each) on gene expression in LPS-activated RAW macrophages; Concentrations of BSP are given in terms of its approximate PEITC content; Effect of any treatment on a specific gene expression was measured by the mRNA quantity relative to the response to LPS activation only (positive control) that is normalized to a value of 1.00. Lower values represent greater inhibitory effects with 0.00 corresponding to a complete inhibition of the induced gene expression. Values are mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$ (post ANOVA comparison with LPS-treated positive control) (A) COX2 assay, (B) iNOS assay, (C) IL1β assay

Figure 5. Comparison between PEITC and rofecoxib treatments (3 replicates for each) on gene expression in LPS-activated RAW macrophages in a time-dependent manner over 21 h following treatments at 5 μM; Effect of any treatment on a specific gene expression was measured by the mRNA quantity relative to the response to LPS activation only (positive control) that is normalized to a value of 1.00. Values are mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$ (significance determined with respect to LPS treated positive control) (A) COX2 assay, (B) iNOS assay, (C) IL1β assay

Figure 6. Effects of PEITC and other isothiocyanates (Phenyl isothiocyanate, Tertiary-butyl isothiocyanate, Isopropyl isothiocyanate, Allyl isothiocyanate) on the expression of the

inflammation-related genes in the LPS-stimulated RAW264.7 macrophages (3 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 was not obtained for Allyl isothiocyanate at 10 μM due to cytotoxicity (A) COX2 assay, (B) iNOS assay, (C) IL1β assay

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FIGURE 1

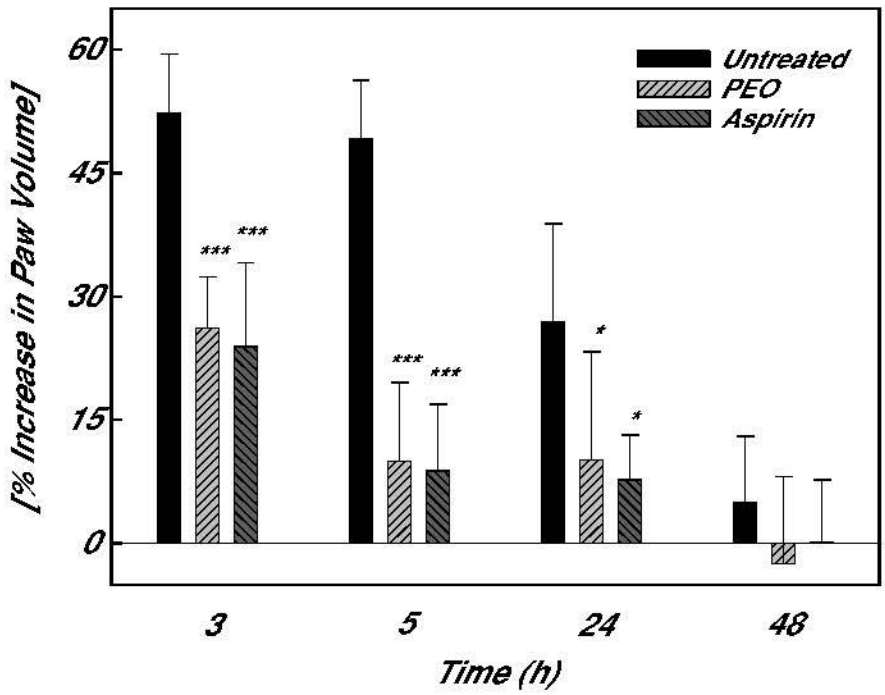

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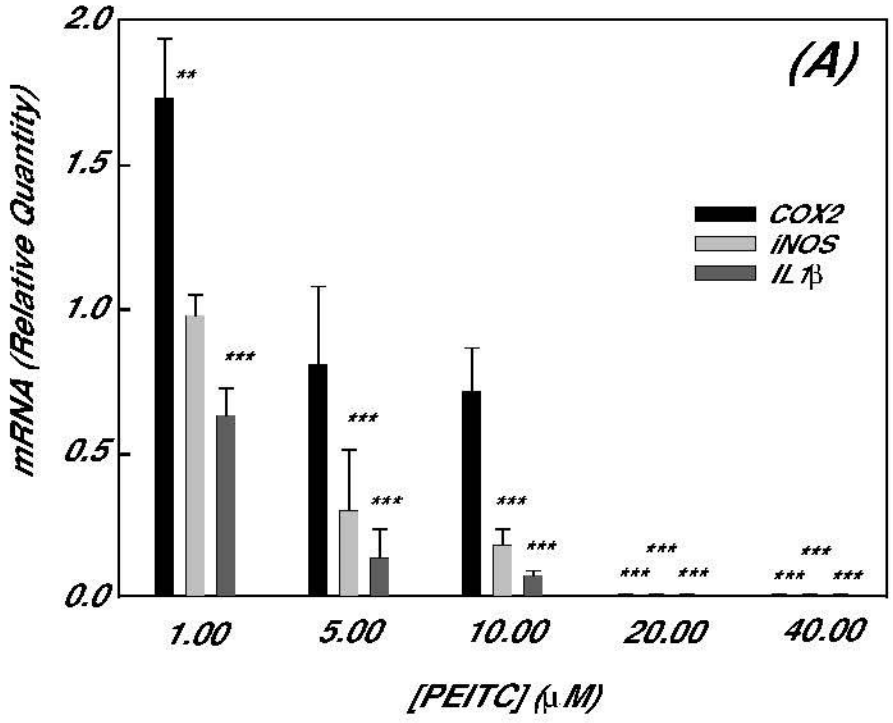
FIGURE 2



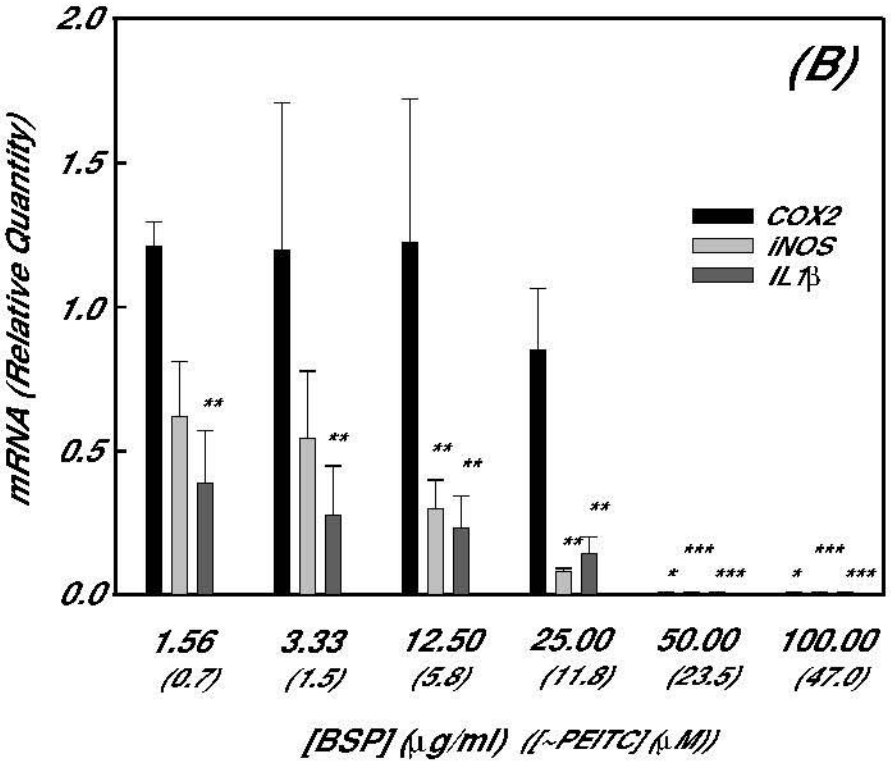


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FIGURE 3

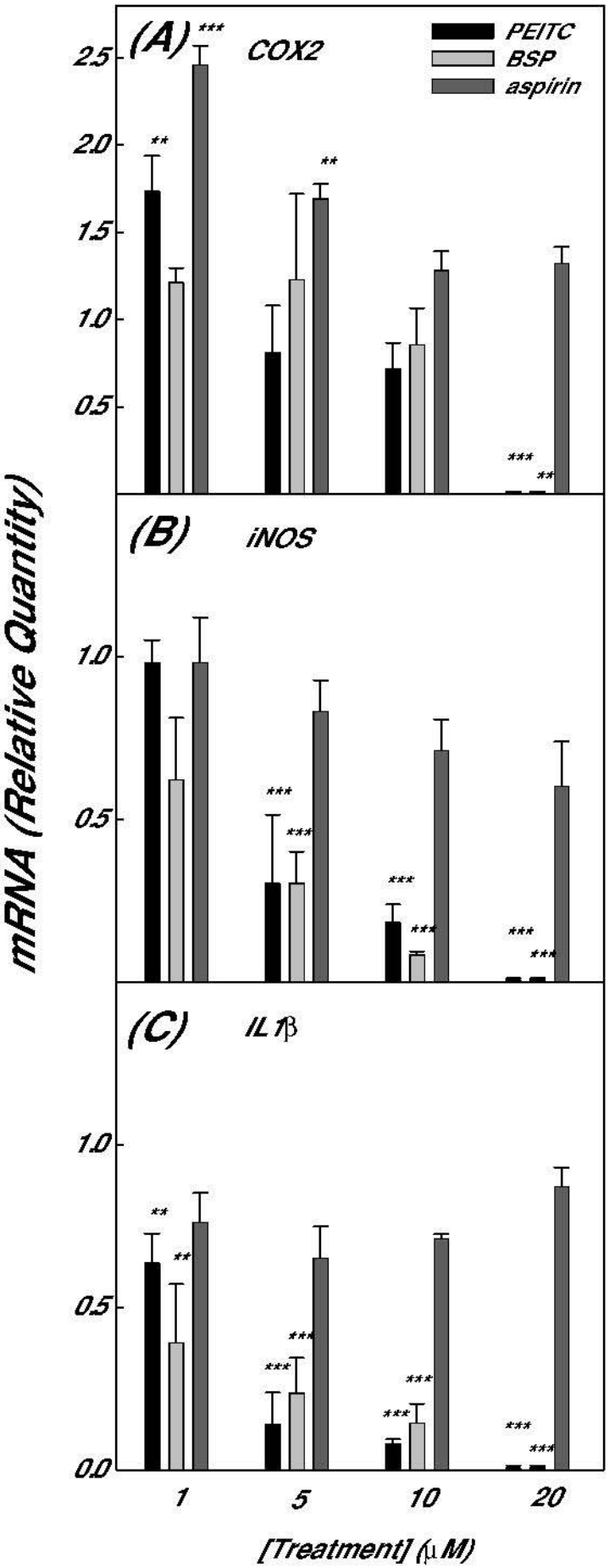

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FIGURE 4

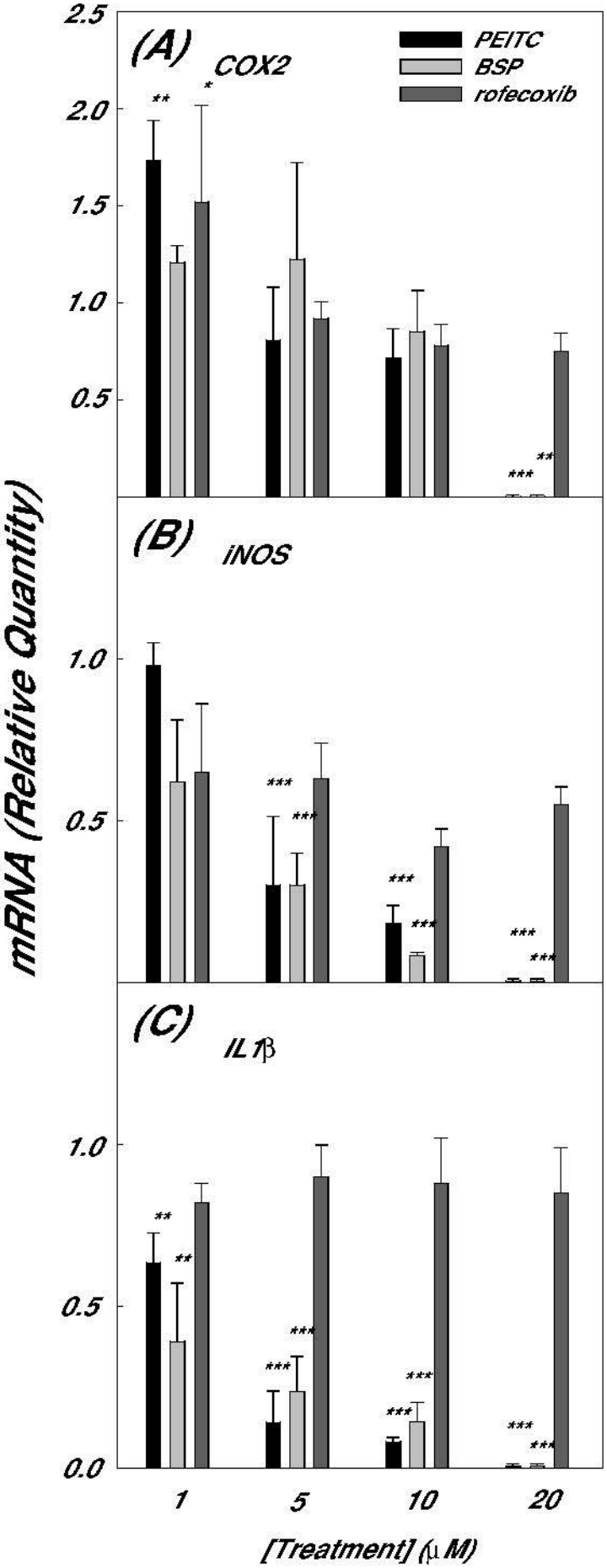

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FIGURE 5

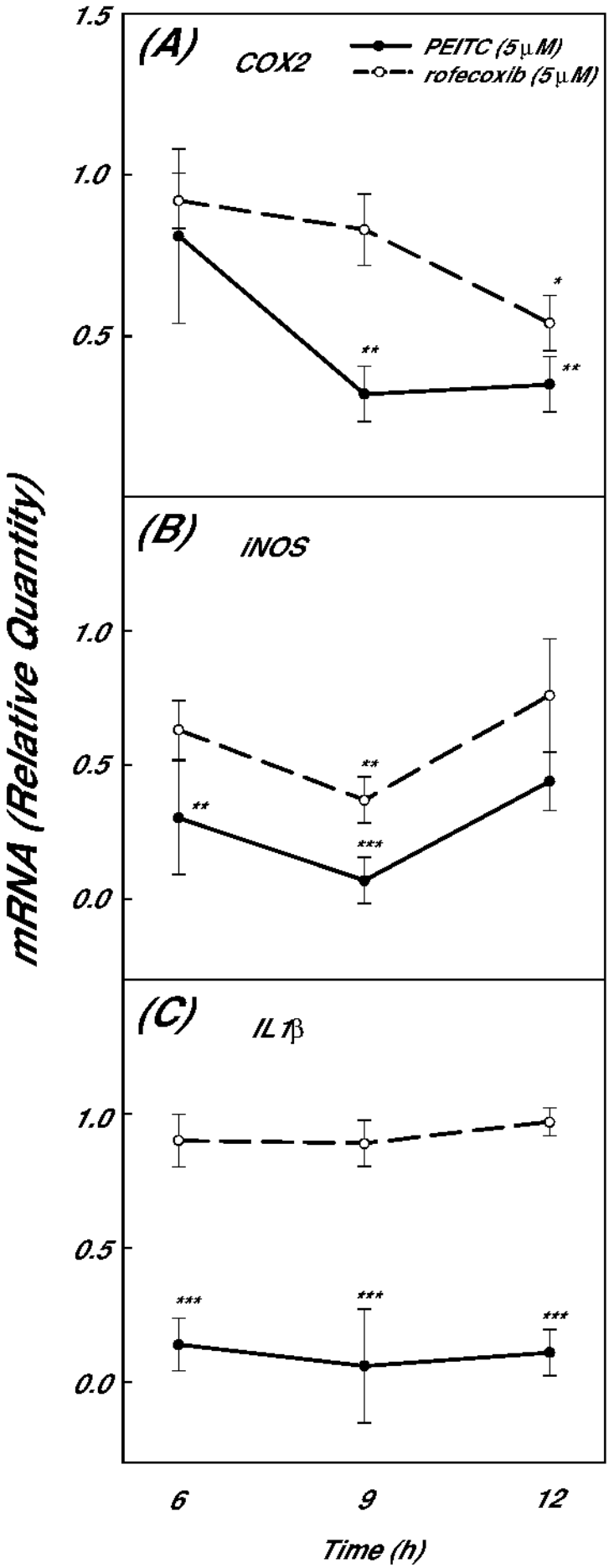

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FIGURE 6