RESEARCH PAPER

Flavocoxid, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, blunts pro-inflammatory phenotype activation in endotoxin-stimulated macrophages

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Background and purpose: The flavonoids, baicalin and catechin, from Scutellaria baicalensis and Acacia catechu, respectively, have been used for various clinical applications. Flavocoxid is a mixed extract containing baicalin and catechin, and acts as a dual inhibitor of cyclooxygenase (COX) and 5-lipoxygenase (LOX) enzymes. The anti-inflammatory activity, measured by protein and gene expression of inflammatory markers, of flavocoxid in rat peritoneal macrophages stimulated with Salmonella enteritidis lipopolysaccharide (LPS) was investigated.

Experimental approach: LPS-stimulated (1 μg/mL) peritoneal rat macrophages were co-incubated with different concentrations of flavocoxid (32–128 μg·mL⁻¹) or RPMI medium for different incubation times. Inducible COX-2, 5-LOX, inducible nitric oxide synthase (iNOS) and inhibitory protein kB-α (kB-α) levels were evaluated by Western blot analysis. Nuclear factor kB (NF-kB) binding activity was investigated by electrophoretic mobility shift assay. Tumour necrosis factor-α (TNF-α) gene and protein expression were measured by real-time polymerase chain reaction and enzyme-linked immunosorbent assay respectively. Finally, malondialdehyde (MDA) and nitrite levels in macrophage supernatants were evaluated.

Key results: LPS stimulation induced a pro-inflammatory phenotype in rat peritoneal macrophages. Flavocoxid (128 μg·mL⁻¹) significantly inhibited COX-2 (LPS = 18 ± 2.1; flavocoxid = 3.8 ± 0.9 integrated intensity), 5-LOX (LPS = 20 ± 3.8; flavocoxid = 3.1 ± 0.8 integrated intensity) and iNOS expression (LPS = 15 ± 1.1; flavocoxid = 4.1 ± 0.4 integrated intensity), but did not modify COX-1 expression. PGE₂ and LTB₄ levels in culture supernatants were consequently decreased. Flavocoxid also prevented the loss of kB-α protein (LPS = 1.9 ± 0.2; flavocoxid = 7.2 ± 1.6 integrated intensity), blunted increased NF-kB binding activity (LPS = 9.2 ± 2; flavocoxid = 2.4 ± 0.7 integrated intensity) and the enhanced TNF-α mRNA levels (LPS = 8 ± 0.9; flavocoxid = 1.9 ± 0.8 n-fold/β-actin) induced by LPS. Finally, flavocoxid decreased MDA, TNF and nitrite levels from LPS-stimulated macrophages.

Conclusion and implications: Flavocoxid might be useful as a potential anti-inflammatory agent, acting at the level of gene and protein expression.


Keywords: flavocoxid; Limbrel; medical food; COX; 5-LOX; iNOS; NF-kB; TNF-α; dual inhibitor; inflammation

Abbreviations: COX, cyclooxygenase; kB-α, inhibitory protein kB-α; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; LTB₄, leukotriene B₄; MAL, malondialdehyde; NF-kB, nuclear factor kB; PGE₂, prostaglandin E₂; TNF-α, tumour necrosis factor-α

Introduction

Inflammation is a beneficial host response to external challenge or cellular injury that leads to the release of a complex array of inflammatory mediators, promoting the restoration of tissue structure and function. However, prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases. During inflammation, fatty acid production is considerably increased (Kuehl and Egan, 1980). Arachidonic acid (AA) is the main precursor of fatty acid metabolites, which regulate functions of various organs and systems. It is released from cellular membrane phospholipids by phospholipase A2 or indirectly by phospholipases C and D (Burdan et al., 2006), and subsequently transformed by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to prostaglandins (PGs), thromboxane and leukotrienes (LTs) (Khanapure et al., 2007).
Both COX and LOX pathways are of particular clinical relevance. COX exists in two distinct isoforms, COX-1 and COX-2, the latter being inducible and primarily involved in inflammation and cell proliferation (Botting, 2006). There is also a third isoform, COX-3, which is a splicing variant of COX-1 with an additional 34 amino acids. This variant has been cloned, partially characterized and appears to be susceptible to inhibition by analgesic compounds such as paracetamol (acetaminophen) (Botting, 2006). Other variants exist, but have not been well characterized specifically in vivo. In recent years, selective COX-2 inhibitors that are as efficacious as traditional non-steroidal anti-inflammatory drugs (NSAIDs), but minimize the risk of unwanted gastrointestinal side effects have been developed (Hinz and Brune, 2002). Unfortunately, a new set of side effects have been identified for this class of NSAIDs related to kidney and cardiovascular dysfunction (Sanghi et al., 2006). Both traditional NSAIDs and the newer coxibs directly inhibit the COX enzymes affecting AA metabolism, but emerging information has suggested an important role of another AA metabolic pathway, 5-LOX and its effect on inflammatory response (Claria and Romano, 2005). In addition, it is becoming clear that NSAIDs shunt AA metabolism towards the 5-LOX pathway and synthesis of LT (Maxix et al., 2006).

The 5-LOX metabolic pathway produces both vasoconstrictive LTs such as LTC4, LTD4 and LTE4 best recognized for their chemotactic properties (Claria and Romano, 2005). Its effect on inflammatory response (Claria and Romano, 2005). In addition, it is becoming clear that NSAIDs shunt AA metabolism towards the 5-LOX pathway and synthesis of LT (Maxix et al., 2006).

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In preliminary experiments, cell viability following exposure to the extract was determined after 24 h of incubation with flavocoxid at various concentrations (from 0.5 to 512 μg·mL⁻¹) or with 1 μg·mL⁻¹ LPS at 37°C. The cells were centrifuged twice and resuspended in the same medium at a concentration of 1 × 10⁶. Macrophages were obtained after 2 h adhesion to plastic Petri dishes (Nunc, Roskilde, Denmark) at 37°C. The homogeneity and viability of the macrophages were greater than 98% as determined by differential staining and trypan blue exclusion. Macrophages were stimulated for 1, 4 or 24 h with 1 μg·mL⁻¹ of LPS. LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL⁻¹) or RPMI medium alone.

Cell viability

In preliminary experiments, cell viability following exposure to the extract was determined after 24 h of incubation with flavocoxid at various concentrations (from 0.5 to 512 μg·mL⁻¹) or with 1 μg·mL⁻¹ LPS at 37°C, by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described by Mosmann (1983).
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Isolation of cytoplasmatic and nuclear proteins
Briefly, total cellular protein was extracted in lysis buffer [25 mM Tris–HCl (pH 7.4), 1.0 mM ethylene glycol tetraacetic acid (EGTA), 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenyl methylsulphonyl fluoride] with added protease and phosphatase inhibitors [100 mM Na3VO4, aprotinin, leupeptin, pepstatin (10 µg·mL−1 each)]. The cell lysate was subjected to centrifugation at 13000 g for 15 min. The supernatant was collected and used for protein concentration determination by Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA). The pellets after a single wash with the hypotonic buffer [10 mM HEPES (pH 7.9), 10.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulphonyl fluoride, aprotinin, leupeptin, pepstatin A (10 µg·mL−1 each) and Na3VO4 (100 mM)] were suspended in an ice-cold buffer with salt [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, protease and phosphatase inhibitors], incubated on ice for 30 min, mixed frequently and centrifuged at 8000 g for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of nuclear proteins was determined by Bio-Rad protein assay.

Western blot of inducible nitric oxide synthase (iNOS), COX-2, 5-LOX and IκB-α
Protein samples (30 µg) were denatured in reducing buffer [62 mM Tris–HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 0.003% bromophenol blue] and separated by electrophoresis on an SDS (12%) polyacrylamide gel. The separated proteins were transferred onto a nitrocellulose membrane using the transfer buffer [39 mM sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS), 5% acrylamide, 0.0625% sodium dodecyl sulphate (SDS)], incubated on ice for 30 min, mixed frequently and centrifuged at 8000 g for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of nuclear proteins was determined by Bio-Rad protein assay.

RNA extraction and real-time polymerase chain reaction (PCR)
Total RNA was isolated using Trizol Reagent (Invitrogen, Milan, Italy), and the procedure was performed according to the protocol provided by the manufacturer. RNA (5 µg) from each sample was reverse transcribed using High-Capacity cDNA Archive Kit according to the manufacturer’s procedures (Applied Biosystems, Foster City, CA, USA). cDNA from each sample (5 ng) was amplified by real-time PCR with 2× TaqMan universal PCR Mastermix (Applied Biosystems), 20× target primer and probe. β-Actin was used as the control as its constitutive expression as a housekeeping gene. Each sample was analysed in duplicates using SDS 7300 (Applied Biosystems). The results were expressed as an n-fold difference relative to normal controls (relative expression levels).

Determination of tumour necrosis factor-α (TNF-α)
TNF-α levels in macrophage culture medium were quantified by ELSA (Bio-Source, Nivelles, Belgium) according to the manufacturer’s instructions.

Nitrite production
Nitrite concentration was measured in a standard Griess reaction. Briefly, 100 µL of supernatant was incubated with an
equal volume of Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid). After 10 min of incubation at room temperature, the absorbance of the chromophore was measured at 540 nm using a microtitre plate reader. Nitrite concentrations were calculated by comparison with a standard calibration curve with sodium nitrite (NaNO₂: 1.26 to 100 μmol·L⁻¹), with control baseline supernatant as the blank.

**Lipid peroxidation**

Lipid peroxidation was measured by malondialdehyde assay. The assay was carried out by using a colorimetric commercial kit (Lipid Peroxidation Assay kit, cat#437634; Calbiochem-Novabiochem Corp., Darmstadt, Germany). Briefly, 0.65 mL 10.3 nM N-methyl-2-phenylindole in acetonitrile was added to 0.2 mL of macrophage supernatant. After vortexing for 3–4 s and adding 0.15 mL of 37% (v/v) HCl, the samples were mixed and closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice, and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard malondialdehyde (MAL) solution (from 2 to 128 nM·mL⁻¹) was also run for comparison.

**Statistical analysis**

All data are expressed as the mean ± SD. Data were analysed by analysis of variance for multiple comparisons of results. The Duncan multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance.

**Drugs**

LPS was obtained from Sigma (Milan, Italy). RPMI 1640 was obtained from Invitrogen. Flavocoxid (composition: >90% purified mixture of baicalin and catechin at a ratio of approximately 4.5:1 with the remainder being excipient (5–6%) and water ~3%) was a kind gift from Primus Pharmaceuticals, Inc. All substances were prepared fresh daily and dissolved in RPMI.

**Results**

**Peritoneal macrophage viability**

To test and verify that the effects of flavocoxid were not due to cytotoxicity, we examined cell viability using the MTT assay. As shown in Figure 1, treatment with flavocoxid, in concentrations up to 128 μg·mL⁻¹, did not impair cell viability as compared with LPS-treated controls. More specifically, the concentration range between 32 and 128 μg·mL⁻¹ caused a significant enhancement in cell viability (Figure 1). Cell viability decreased slightly at 256 and 512 μg·mL⁻¹ (~50%). These data prompted us to test flavocoxid at the concentrations of 32, 64 and 128 μg·mL⁻¹ in all experiments. These values are within the concentration ranges already shown to inhibit both COX-1 and COX-2 peroxidase enzyme activities and 5-LOX activity in vitro (Burnett et al., 2007; Pillai and Burnett, unpubl. experiments).

**Flavocoxid reduces COX-1, COX-2 and 5-LOX expression**

COX-1 was constitutively expressed at low levels and did not show induction with LPS (Figure 2A). COX-2 and 5-LOX enzymes were also present in control macrophages (Figure 2B,C) at very low levels. The upper panels of Figure 2A–C show representative Western blot analysis. The lower panels represent quantitative data. LPS stimulation of peritoneal macrophages for 24 h resulted in a marked increase in expression of both COX-2 and 5-LOX. Treatment of cell cultures with flavocoxid significantly attenuated, in a concentration-dependent manner, the increase in COX-2 and 5-LOX expression (Figure 2B,C), but not COX-1 expression (Figure 2A).

**Flavocoxid reduced LPS-stimulated LTB₄ and PGE₂ production in peritoneal macrophages**

Both LTB₄ and PGE₂ were present in supernatants from control macrophages (Figure 3A,B) at low levels. Together with LPS-induced COX-2 and 5-LOX activation, the production of PGE₂ and LTB₄ was augmented. Treatment with flavocoxid significantly attenuated, in a concentration-dependent manner, this increase in PGE₂ and LTB₄ (Figure 3A,B).

**Flavocoxid inhibits the cytoplasmic loss of IkB-α protein and decreases the activation of NF-κB in peritoneal macrophages**

NF-κB activation in the nuclear extracts of peritoneal macrophages was determined by EMSA 1 h after LPS challenge. The upper panel of Figure 4A shows representative EMSA indicating activation of NF-κB. The lower panel of Figure 4A shows quantitative data for the shift of radio-labelled protein. NF-κB binding activity was present at very low levels in control macrophages. In contrast, LPS stimulation increased NF-κB binding activity.
binding activity over threefold (Figure 4A). The administration of flavocoxid markedly reduced NF-κB binding activity down to baseline levels at 128 μg·mL⁻¹ (Figure 4A).

The NF-κB inhibitory protein inhibitor IκBα (IκB-α) was also investigated 1 h following LPS challenge. The upper panel of Figure 4B shows representative Western blot analysis indicating the presence of IκB-α protein. The lower panel of Figure 4B represents quantitative data for protein expression of IκB-α. LPS treatment induced a significant degradation of the protein. In contrast, loss of cytoplasmic IκB-α was blocked by flavocoxid restoring baseline levels of IκB-α at 128 μg·mL⁻¹ (Figure 4B).

Flavocoxid inhibits iNOS activation and nitrite production in peritoneal macrophages

No significant iNOS expression or measurable nitrite levels were observed in control macrophages (Figure 5A,B). Stimulation, with LPS for 24 h, of peritoneal macrophages resulted in a marked increase of iNOS expression of over threefold and in an augmented release of nitrite. Treatment with flavocoxid significantly blunted, in a concentration-dependent manner, the increase in iNOS expression and nitrite release (Figure 5A,B).

Flavocoxid reduces TNF-α expression and production

TNF-α mRNA and mature protein were constitutively present in control macrophages at low levels (Figure 6A,B). Stimulation (4 h) of peritoneal macrophages caused a robust increase in TNF-α message and protein expression. Flavocoxid produced a significant and concentration-dependent reduction in the levels of TNF-α mRNA and in the release of the mature protein into the supernatant (Figure 6A,B).

Flavocoxid reduces MAL levels in peritoneal macrophages

Very low MAL levels were present in control macrophages. Levels of this by-product of the oxidation of AA were...
evaluated following a 1 h LPS stimulation which showed a marked increase in MAL content (Figure 7). Flavocoxid addition produced a significant concentration-dependent decrease in the levels of MAL in LPS-stimulated macrophages, suggesting a direct antioxidant effect on the production of MAL from AA (Figure 7).

Discussion and conclusions

Recently, the plant kingdom has received increased attention as a source of therapeutic molecules (Wagner, 1993; Bauer, 1999). Flavonoids are polyphenolic compounds which are integral components of the human diet found in all fruits and vegetables. Several plants and spices containing flavonoid derivatives have found application as disease-preventive and therapeutic agents in traditional medicine in Asia for thousands of years (Kandaswami et al., 2005). Our present experiments used a standardized, proprietary mixture of two flavanoids, baicalin and catechin, called flavocoxid (marketed as Limbrel).

In unpublished experiments, Pillai and Burnett have found that flavocoxid inhibited purified ovine COX-1 and COX-2 enzymes at approximately equivalent IC₅₀ values, yielding a ratio for COX-1/COX-2 of 1.3. This value was close to that determined for ibuprofen (1.31) and distinct from the ratios for COX-2 selective inhibitors (rofecoxib, 625; valdecoxib, 81; celecoxib, 10), in the same assay. However, only flavocoxid inhibited the 5-LOX enzyme. Although these results illustrate the direct effect of
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flavocoxid on AA metabolism, they do not address the possibility of regulation of gene or protein expression of inflammatory markers.

To test whether flavocoxid could affect specific inflammatory markers involved in chronic disease, a peritoneal macrophage model stimulated with LPS was used. Baicalin is known to reduce the viability of cancer cell lines involving an apoptotic mechanism via a mitochondrial pathway (Ueda et al., 2002). Jurkat cell viability, for example, was reduced by 100% between 100 and 400 μg·mL⁻¹ baicalin, but only slightly affected peripheral blood monocytes. Viability of other cancer cells, such as prostate and bladder, is significantly reduced at concentrations between 30 and 100 μg·mL⁻¹ (Adams et al., 2006; Chao et al., 2007). Tea catechins also show the same propensity to dramatically reduce cell viability in immortalized cell lines at concentrations that did not affect macrophages in our work (Mittal et al., 2004). Catechin has also been shown to protect keratinocytes from cell death induced by UV light (Wu et al., 2006). Indeed, murine RAW264.7 cells show increased viability when stimulated with 1 μg·mL⁻¹ LPS even in the presence of baicalin (Liu et al., 2008). Based on this evidence, the observed cell death of less than 50% at 256 and 512 μg·mL⁻¹ is much milder compared to the effects of baicalin or catechin on other cell types at almost 5- to 10-fold lower concentrations, representing an acceptable margin of error to test flavocoxid on NF-kB-induced inflammatory gene and protein markers.

A variety of flavonoids are known to modulate inflammation by affecting NF-kB levels primarily through their antioxidant activities (Nam, 2006; Rodrigo and Bosco, 2006). Indeed, both baicalin and catechin, as well as epicatechin, its enantiomer, have been found to have potent antioxidant activities (Lotito and Fraga, 2000; Shieh et al., 2000). In agreement with this idea, flavocoxid significantly reduced oxidative stress/lipid peroxidation in rat peritoneal macrophages challenged with LPS by reducing MAL levels in cell culture. The antioxidant capacity of flavocoxid has been confirmed in vitro exhibiting a total oxygen radical absorbance capacity score, approximately twice that of vitamin C and more than three times greater than vitamin E (Pillai and Burnett, unpubl. experiments). Control of oxidative stress may also account for the observed gene expression and protein down-regulation by flavocoxid via modulation of NF-kB and IκB-α.

Oxidative stress can trigger an inflammatory cascade in which NF-kB plays a prominent role (Pandito et al., 2006). NF-kB is an important transcription factor complex that regulates the expression of many genes involved in immune and inflammatory responses in chronic human disease (Kopp and Ghosh, 1995; Baldwin, 2001). In unstimulated cells (Baldwin, 1996), NF-kB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called IκB-α. Following activation, the NF-kB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes, including the genes encoding the pro-inflammatory cytokines; adhesion molecules; chemokines; and inducible enzymes such as COX-2, 5-LOX and iNOS (Pahl, 1999), but not COX-1 as reflected in our results. Our data indicate that flavocoxid reduced IκB-α loss from the cytoplasm and NF-kB binding to DNA in LPS-stimulated macrophages. Flavocoxid-mediated NF-kB modulation resulted in a direct down-regulation of COX-2 protein production in culture in agreement with data on periodontal ligament cells in vitro in which baicalin reduced NF-kB-mediated COX-2 production (Wang et al., 2006). Celecoxib produces the opposite effect inducing COX-2 expression in inflamed animal model (Niederberger et al., 2001). Tea catechins, composed of gallate esters of catechin, have also been shown to modulate COX-2 gene expression in colon carcinoma cells in vitro (Mutioh et al., 2000). Baicalin was also found to protect rat cortical neurons against ischaemic injury by attenuating the production of 5-LOX (Ge et al., 2007), similar to our present observations with peritoneal macrophages.

Activation of NF-kB during inflammation regulates the expression of almost 400 different genes, which include enzymes such as the iNOS which is involved in inflammation and cellular injury. Activated macrophages

Figure 6 Tumour necrosis factor-α (TNF-α) mRNA (A) and TNF-α levels (B) in macrophages stimulated for 4 h with 1 μg·mL⁻¹ of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL⁻¹) or RPMI alone. Bars represent the mean ± SD of seven experiments. *P < 0.05 versus control, #P < 0.01 versus LPS + RPMI, ##P < 0.005 versus LPS + RPMI, $P < 0.001 versus LPS + RPMI.

Figure 7 Malondialdehyde (MAL) levels in macrophages stimulated for 4 h with 1 μg·mL⁻¹ of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL⁻¹) or RPMI alone. Bars represent the mean ± SD of seven experiments. *P < 0.05 versus control, #P < 0.01 versus LPS + RPMI, ##P < 0.005 versus LPS + RPMI, $P < 0.001 versus LPS + RPMI.
transcriptionally express iNOS, which catalyses the oxidative deamination of L-arginine to produce NO, and is responsible for prolonged and profound production of NO (Xie and Nathan, 1994). High output of NO by iNOS can provoke deleterious consequences such as septic shock and other inflammatory diseases (Zamora et al., 2000). NO production by iNOS is regulated mainly at the transcriptional level, and the expression of the iNOS gene in macrophages is under the control of several transcription factors, which include NF-κB (Aktan, 2004). The presence of a cis-acting NF-κB element has been demonstrated in the S′ flanking regions of iNOS genes (Lowenstein et al., 1993). In agreement with this hypothesis, our results show that flavocoxid attenuated the increase in either iNOS expression or NO content, probably through NF-κB activity inhibition, and may block the amplifying loop of the inflammatory response involving iNOS and NO production. Tea catechins have been shown to attenuate iNOS and NO production as well (Lin and Lin, 1997), but our results are in contrast to an earlier report that baicalin did not affect iNOS activity in LPS-stimulated mouse peritoneal macrophages (Liu et al., 2008). It is possible that the iNOS attenuation is due to the catechin component of flavocoxid or synergistic effects of both molecules. The inhibitory activity of flavocoxid on AA metabolism (Burnett et al., 2007; Pillai and Burnett, unpubl. experiments), expression of COX-2 and 5-LOX, as well as inhibition of iNOS production of NO, fits with a proposed ‘cross-talk’ model between NO production and AA metabolic effects which occur in inflammation (Mariotto et al., 2007). More study, however, is needed to elucidate this connection.

TNF-α is another NF-κB-inducible product that has an early and crucial role in the cascade of pro-inflammatory cytokine production and subsequent inflammatory processes (Cunha et al., 1992). Flavocoxid produced a significant reduction in the levels of TNF-α mRNA and in the release of the mature protein, confirming an earlier study on baicalin alone decreasing the production of cytokines in cultured peripheral blood monocytes (Krahauer et al., 1999). Catechin and its oligomeric cousin, procyanidin, from cocoa extracts have previously been shown to decrease TNF-α secretion from resting and phytohaemagglutinin-stimulated human peripheral blood monocytes in agreement with our data (Mao et al., 2002). This is in contrast to celecoxib which induced TNF-α production in a yzomosan-induced inflammation model in rats suggesting an up-regulation of NF-κB (Niederberger et al., 2001). Celecoxib has been shown, however, to inhibit inflammatory gene expression signal in the p38 pathway (Tegeder et al., 2001), as found for flavocoxid (Messina et al. unpubl. data).

The current gene expression and protein production data on inflammatory markers along with previous metabolite data relating to dual inhibition of AA metabolism suggest that flavocoxid acts via a variety of mechanisms to inhibit inflammation, making it a promising therapeutic agent for OA, as well as other inflammatory musculoskeletal disorders. Only through further experimental animal models and human clinical trials, however, will we be able to ascertain whether flavocoxid may represent a new option for treatment of other chronic diseases states with inflammation as part of their aetiology.

Conflict of interest

Dr Bruce Burnett works for Primus Pharmaceuticals, Inc., the manufacturer of flavocoxid. The other authors state no conflict of interest.

References


