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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 $\mu g \cdot mL^{-1}$) peritoneal rat macrophages were co-incubated with different concentrations of flavocoxid (32–128 $\mu g \cdot mL^{-1}$) or RPMI medium for different incubation times. Inducible COX-2, 5-LOX, inducible nitric oxide synthase (iNOS) and inhibitory protein κB-α (IκB-α) levels were evaluated by Western blot analysis. Nuclear factor κB (NF-κB) 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 $\mu g \cdot mL^{-1}$) 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. PGE_2 and LTB_4 levels in culture supernatants were consequently decreased. Flavocoxid also prevented the loss of IκB-α protein (LPS = 1.9 ± 0.2; flavocoxid = 7.2 ± 1.6 integrated intensity), blunted increased NF-κB 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.

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

Abbreviations: COX, cyclooxygenase; IκB-α, inhibitory protein κB-α; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; LTB_4, leukotriene B_4; MAL, malondialdehyde; NFκB, nuclear factor κB; PGE_2, prostaglandin E_2; 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)

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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 (Clària and Romano, 2005). In addition, it is becoming clear that NSAIDs shunt AA metabolism towards the 5-LOX pathway and synthesis of LT (Maxis *et al.*, 2006).

The 5-LOX metabolic pathway produces both vasocontrictive LTs such as LTC_4, LTD_4 and LTE_4 best recognized for their roles in bronchoconstriction (Peters-Golden, 2008), as well as chemoattractant LTB_4 which attracts leucocytes to the site of tissue damage to perform needed repair functions (Ford-Hutchinson *et al.*, 1980; Yokomizo *et al.*, 1997). NSAID-induced gastric inflammation has been associated with shunting of the AA to generate LTB_4 from 5-LOX (Hudson *et al.*, 1993; Celotti and Durand, 2003). In cardiovascular animal disease models treated with COX pathway inhibitors, 5-LOX production of LT from cells present in plaques has been shown to promote inflammation in endothelial cells via the influx of leucocytes and vasoconstriction of arteries (Mehrabian *et al.*, 2002; Bäck, 2008). And in the kidney, chronic use of COX inhibitors leads to renal dysfunction (Perazella and Eras, 2000), and damage is partly mediated by an up-regulation of 5-LOX and LTB_4-induced influx of leucocytes (Maccarrone *et al.*, 1999). The shunting of AA metabolism down the 5-LOX pathway may therefore add or exacerbate adverse events while a patient is taking a traditional NSAID or selective COX-2 inhibitor.

Inhibition of the biosynthesis of such inflammatory mediators by blocking the AA-processing activities of COX and 5-LOX is considered as a promising approach to manage osteoarthritis (OA) (Celotti and Laufer, 2001). Recently, reports have appeared regarding so-called 'dual inhibitors', agents that inhibit not only COX-1 and COX-2, but also 5-LOX-mediated AA metabolism (Celloti and Durand, 2003; Shelly and Hawkey, 2003; Brune, 2004). These agents may be particularly effective for minimizing both gastric and cardiovascular side effects by balancing AA metabolism in the body (Martel-Pelletier *et al.*, 2003; Leone *et al.*, 2007).

Flavonoids from *Scutellaria baicalensis* (Chinese skullcap) and *Acacia catechu* (black catechu) have been used in many traditional medicines and pharmaceutical products for a variety of purposes, including anti-inflammatory, antiviral, antibacterial, anticancer and cardiovascular applications (van Loon, 1997; Chou *et al.*, 2003; De Clerq, 2005; Huang *et al.*, 2005). Flavocoxid, marketed as an FDA-regulated medical food, Limbrel®, for the clinical dietary management of the metabolic aspects of OA in the United States, is a mixed extract containing the naturally occurring flavonoids, baicalin and catechin, and has been shown to act as a dual inhibitor of COX-1 and COX-2 peroxidase enzyme activities with significant inhibition of 5-LOX enzyme activity *in vitro* (Burnett *et al.*, 2007; Pillai and Burnett, unpubl. experiments). These findings may help explain preliminary *in vivo* data in animals showing that flavocoxid decreases, in a dose-dependent manner, edema in an AA-induced mouse ear swelling model, and abates the swelling and restores function in mice subjected to an intra-articular space injection of AA (Burnett *et al.*, 2007). Flavocoxid has also been shown to be equivalent to naproxen for efficacy as measured by quality of life end points in at least one well-controlled human clinical trial (Levy *et al.*, 2007). These results are, on the surface, direct inhibitory effects on proteins which process AA and thus reduce inflammatory fatty acid metabolites. Although the relative inhibition of COX and 5-LOX AA processing by flavocoxid is known, there are no data on how it affects gene expression and protein levels of inflammatory markers from immune cells. Therefore, the aim of the present study was to investigate the effects of flavocoxid in rat peritoneal macrophages stimulated by lipopolysaccharide (LPS) and its effect on the levels of gene expression and protein markers of inflammation.

Methods

Animals and isolation of peritoneal macrophages

All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). Peritoneal MΦs were obtained from male Sprague–Dawley rats (250–275 g) by washing the abdominal cavity with RPMI 1640. The cells were centrifuged twice and resuspended in the same medium at a concentration of 1×10^6. Macrophages were obtained after 2 h adhesion to plastic Petri dishes (Nunc, Roskilde, Denmark) at 37°C. The homogeneity and the viability of 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 $\mu g \cdot mL^{-1}$ of LPS. LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 $\mu g \cdot mL^{-1}$) 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 $\mu g \cdot mL^{-1}$) or with 1 $\mu g \cdot mL^{-1}$ LPS at 37°C, by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described by Mosmann (1983).

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 Na_3VO_4, aprotinin, leupeptin, pepstatin (10 $\mu g \cdot mL^{-1}$ each)]. The cell lysate was subjected to centrifugation at 1300× *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), 1.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulphonyl fluoride, aprotinin, leupeptin, pepstatin A (10 $\mu g \cdot mL^{-1}$ each) and Na_3VO_4 (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 800× *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 glycine, 48 mM Tris–HCl (pH 8.3), 20% methanol] at 200 mA for 1 h. The membranes were stained with Ponceau S (0.005% in 1% acetic acid) to confirm equal amounts of protein, and blocked with 5% non-fat dry milk in TBS-0.1% Tween-20 for 1 h at room temperature; washed three times for 10 min each in TBS-0.1% Tween-20; and incubated with a primary antibody for iNOS (Chemicon, Temecula, CA, USA), COX-2, COX-1, 5-LOX (Abcam, Cambridge, UK) and IκB-α (Cell Signaling Technology, Inc., Beverly, MA, USA) in TBS-0.1% Tween-20 overnight at 4°C, diluted 1:1000. After being washed three times for 10 min each in TBS-0.1% Tween-20, the membranes were incubated with a specific peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) for 1 h at room temperature diluted 1:20 000. After washing, the membranes were analysed by the enhanced chemiluminescence system according to the manufacturer's protocol (Amersham, Little Chalfont, UK). The protein signals were quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared to controls. β-Actin (Cell Signaling Technology) was used as a comparator using antibody detection on stripped blots to confirm equal protein loading and blotting.

Evaluation of LTB_4 and PGE_2 in cell supernatants

Samples stored at –80°C were assayed for LTB_4 using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA; catalogue number KGE006B) based on the forward sequential competitive binding technique in which LTB_4 present in samples competes with a fixed amount of horseradish peroxidase-labelled LTB_4 for sites on a chicken polyclonal antibody. The absorbance was read at 450 nm. The intensity of the colour was proportional to the concentration of LTB_4 in the sample.

PGE_2 was directly assayed without purification by using the Cayman EIA kit (Cayman, Ann Arbor, MI, USA; catalogue number 514 010). Samples were run in duplicate, and the absorbance was spectrophotometrically read at 412 nm and was directly proportional to the content of PGE_2 in samples.

Determination of NF-κB binding activity

NF-κB binding activity was performed using electrophoretic mobility shift assay (EMSA); 15 μg of nuclear extract was incubated for 30 min at room temperature with 50 fmol of biotin-end-labelled 45-mer double-strand NF-κB oligonucleotide from the HIV long terminal repeat, 5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGAGGCGTGGG-3′ containing 2 NF-κB binding sites (underlined). Both complementary oligonucleotides were end-labelled separately and then annealed prior to use. Binding reaction mixtures were prepared in a final volume of 20 μL HEPES buffer containing 1 mg double-strand poly dI/dC, 10% glycerol, 100 mM $MgCl_2$ and 1% Nonidet P-40, performed with the LightShift Chemiluminescent EMSA Kit (Pierce, Milan, Italy), according to the manufacturer's instructions. Bound complexes were separated on 7.5% non-denaturing polyacrylamide gels, blotted onto nylon membrane and visualized on Kodak X-ray film (Kodak, Milan, Italy) by autoradiography. The EMSA NF-κB binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil Celbio). The results of each group were expressed as relative integrated intensity.

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 due to 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 ELISA (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 ($NaNO_2$: 1.26 to 100 $\mu mol \cdot L^{-1}$), 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 $nM \cdot mL^{-1}$) 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 $\mu g \cdot mL^{-1}$, did not impair cell viability as compared with LPS-treated controls. More specifically, the concentration range between 32 and 128 $\mu g \cdot mL^{-1}$ caused a significant enhancement in cell viability (Figure 1). Cell viability decreased slightly at 256 and 512 $\mu g \cdot mL^{-1}$ (<50%). These data prompted us to test flavocoxid at the concentrations of 32, 64 and 128 $\mu g \cdot mL^{-1}$ in all experiments. These values are within the concentration ranges already shown to inhibit both COX-1 and COX-2 peroxidase enzyme activities

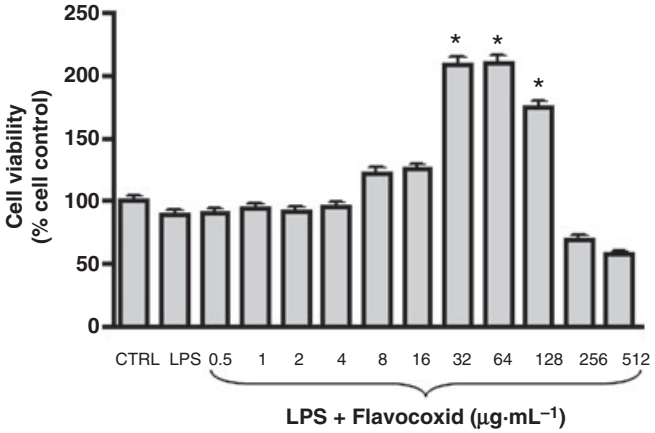

Figure 1 Cell viability in macrophages stimulated for 24 h with 1 $\mu g \cdot mL^{-1}$ of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid or RPMI alone. Bars represent the mean ± SD of seven experiments. *$P < 0.001$ versus LPS.

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 LTB_4 and PGE_2 production in peritoneal macrophages
Both LTB_4 and PGE_2 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 PGE_2 and LTB_4 was augmented. Treatment with flavocoxid significantly attenuated, in a concentration-dependent manner, this increase in PGE_2 and LTB_4 (Figure 3A,B).

Flavocoxid inhibits the cytoplasmic loss of IκB-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

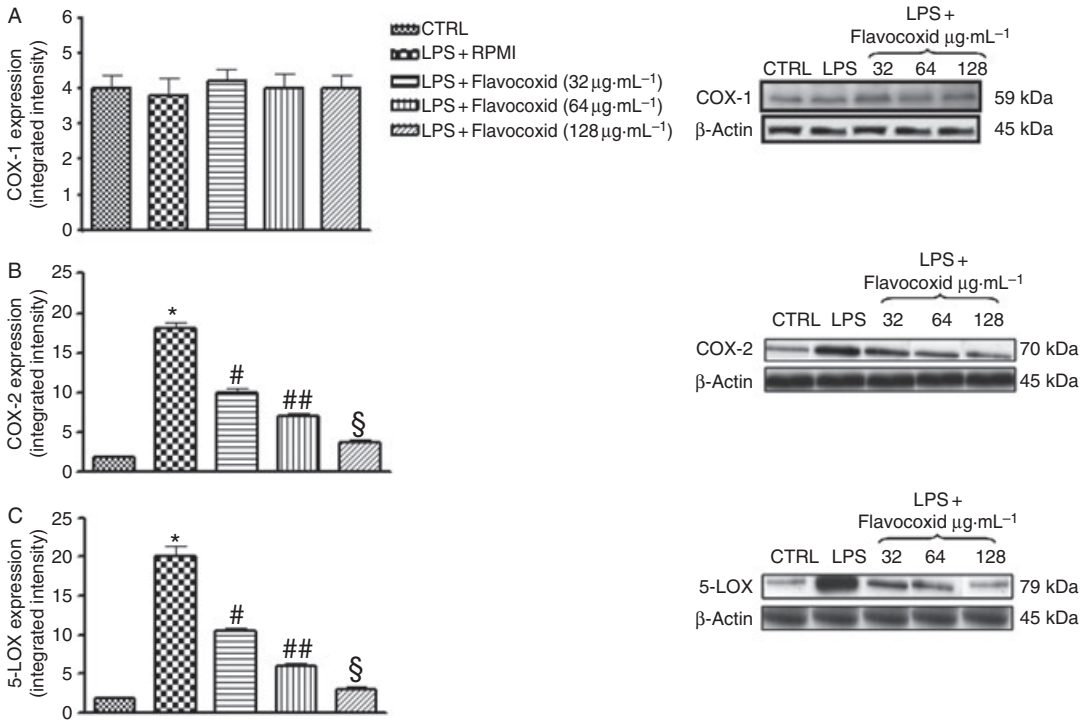

Figure 2 Western blot analysis of COX-1 (A), COX-2 (B) and 5-LOX expression (C) in macrophages stimulated for 24 h with 1 μg·mL^{-1} of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg mL^{-1}) or RPMI alone. Bars represent the mean ± SD of seven experiments. *P < 0.05 versus control, $^{\#}P$ < 0.01 versus LPS + RPMI, $^{\#\#}P$ < 0.005 versus LPS + RPMI, $^{\S}P$ < 0.001 versus LPS + RPMI.

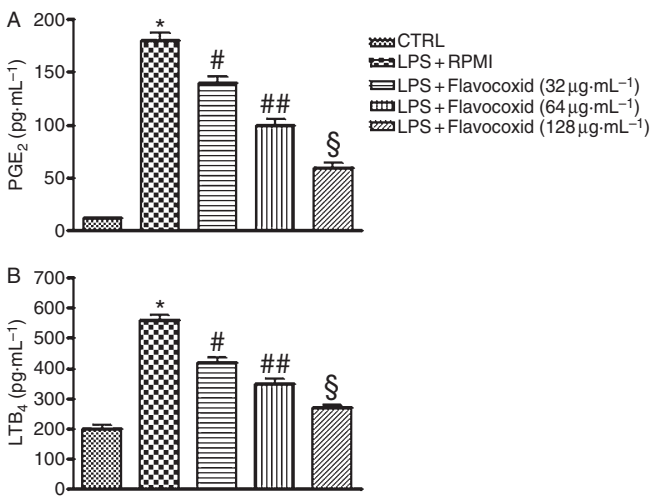

Figure 3 LTB_4 (B) and PGE_2 (C) concentrations in macrophages stimulated for 24 h with 1 μg·mL^{-1} of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL^{-1}) or RPMI alone. Bars represent the mean ± SD of seven experiments. *P < 0.05 versus control, $^{\#}P$ < 0.01 versus LPS + RPMI, $^{\#\#}P$ < 0.005 versus LPS + RPMI, $^{\S}P$ < 0.001 versus LPS + RPMI.

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 κBα (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 Iκ-Bα. 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). LPS 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

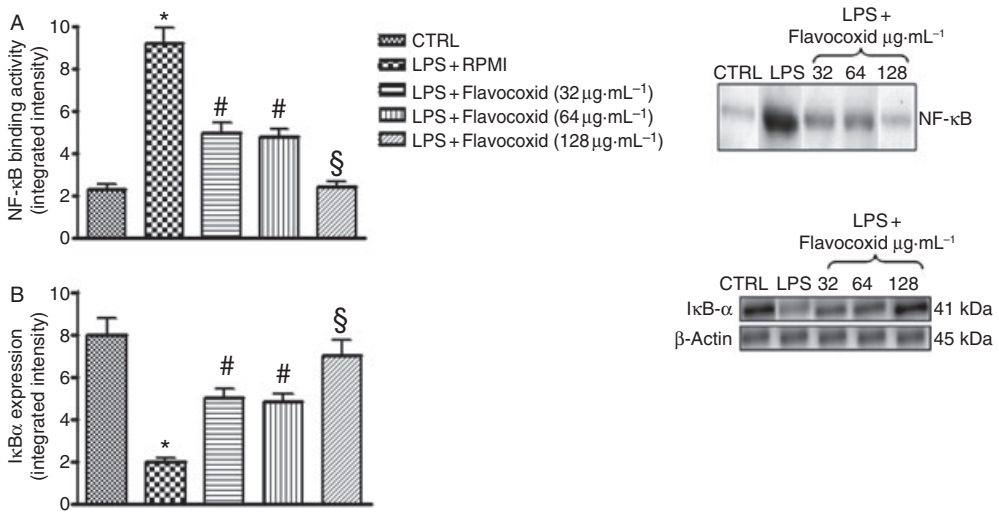

Figure 4 Electrophoretic mobility shift assay (EMSA) of NF-κB binding activity in the nucleus (A) and Western blot analysis of IκB-α proteins levels (B) in the cytoplasm of macrophages stimulated for 1 h with 1 μg·mL^{-1} of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages 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, $^{\S}P$ < 0.001 versus LPS + RPMI.

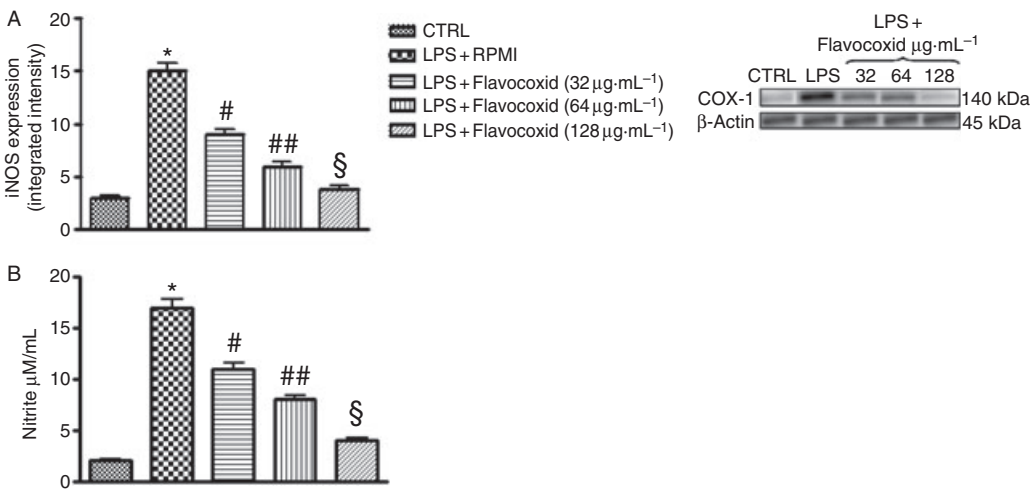

Figure 5 Western blot analysis of inducible nitric oxide synthase (iNOS) activity (A) and nitrite concentration (B) in macrophages stimulated for 24 h with 1 μg·mL^{-1} of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL^{-1}) or RPMI alone. Bars represent the mean ± SD of seven experiments. *P < 0.05 versus control, $^{\#}P$ < 0.005 versus LPS + RPMI, $^{\S}P$ < 0.001 versus LPS + RPMI.

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 IC_{50} values, yielding a ratio for $COX\text{-}1_{IC50}/COX\text{-}2_{IC50}$ 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

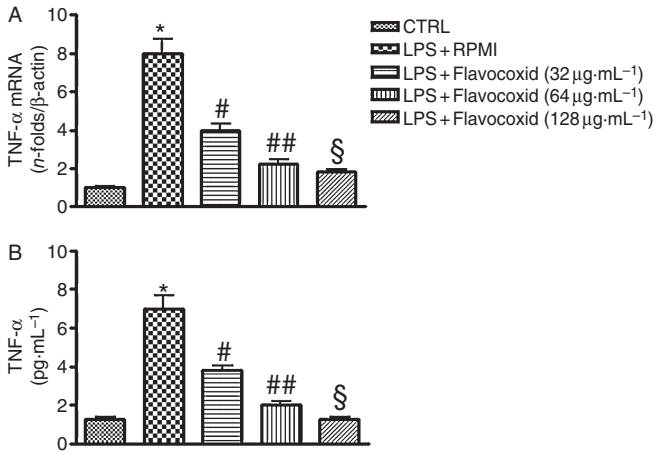

Figure 6 Tumour necrosis factor-α (TNF-α) mRNA (A) and TNF-α levels (B) in macrophages stimulated for 4 h with 1 μg·mL^{-1} of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL^{-1}) or RPMI alone. Bars represent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

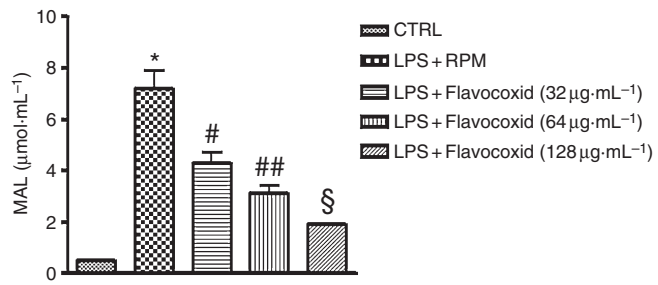

Figure 7 Malondialdehyde (MAL) levels in macrophages stimulated for 4 h with 1 μg·mL^{-1} of lipopolysaccharide (LPS) or its vehicle (1 mL of RPMI). LPS-stimulated macrophages were co-incubated with flavocoxid (32, 64 and 128 μg·mL^{-1}) or RPMI alone. Bars represent the mean ± SD of seven experiments. *P < 0.05 versus control, $^{\#}P$ < 0.01 versus LPS + RPMI, $^{\#\#}P$ < 0.005 versus LPS + RPMI, $^{\S}P$ < 0.001 versus LPS + RPMI.

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 prostrate 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-κB-induced inflammatory gene and protein markers.

A variety of flavonoids are known to modulate inflammation by affecting NF-κB 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-κB and IκB-α.

Oxidative stress can trigger an inflammatory cascade in which NF-κB plays a prominent role (Pantano *et al.*, 2006). NF-κB 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-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called IκB-α. Following activation, the NF-κB 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-κB binding to DNA in LPS-stimulated macrophages. Flavocoxid-mediated NF-κB 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-κB-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* (Muthoh *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-κB 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

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 5′ 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*., 2001). Catechin and its oligomeric cousin, procyanidin, from cocoa extracts have previously been shown to decrease TNF-α secretion from resting and phytohaemaglutinin-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 zymosan-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 metabolic 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.

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