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RESEARCH PAPER

Effects of flavocoxid, a dual inhibitor of COX and 5-lipoxygenase enzymes, on benign prostatic hyperplasia

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BACKGROUND AND PURPOSE

Inflammation plays a key role in the development of benign prostatic hyperplasia (BPH). Eicosanoids derived from the COX and 5-lipoxygenase (5-LOX) pathways are elevated in the enlarging prostate. Flavocoxid is a novel flavonoid–based 'dual inhibitor' of the COX and 5-LOX enzymes. This study evaluated the effects of flavocoxid in experimental BPH.

EXPERIMENTAL APPROACH

Rats were treated daily with testosterone propionate (3 $mg \cdot kg^{-1}$ s.c.) or its vehicle for 14 days to induce BPH. Animals receiving testosterone were randomized to receive vehicle (1 $mL \cdot kg^{-1}$, i.p.) or flavocoxid (20 $mg \cdot kg^{-1}$, i.p.) for 14 days. Histological changes, eicosanoid content and mRNA and protein levels for apoptosis-related proteins and growth factors were assayed in prostate tissue. The effects of flavocoxid were also tested on human prostate carcinoma PC3 cells.

KEY RESULTS

Flavocoxid reduced prostate weight and hyperplasia, blunted inducible expression of COX-2 and 5-LOX as well as the increased production of PGE_2 and leukotriene B_4 (LTB_4), enhanced pro-apoptotic Bax and caspase-9 and decreased the anti-apoptotic Bcl-2 mRNA. Flavocoxid also reduced EGF and VEGF expression. In PC3 cells, flavocoxid stimulated apoptosis and inhibited growth factor expression. Flavocoxid-mediated induction of apoptosis was inhibited by the pan-caspase inhibitor, Z-VAD-FMK, in PC3 cells, suggesting an essential role of caspases in flavocoxid-mediated apoptosis during prostatic growth.

CONCLUSION AND IMPLICATIONS

Our results show that a 'dual inhibitor' of the COX and 5-LOX enzymes, such as flavocoxid, might represent a rational approach to reduce BPH through modulation of eicosanoid production and a caspase-induced apoptotic mechanism.

Abbreviations

BPH, benign prostatic hyperplasia; DHT, dihydrotestosterone; LUTS, lower urinary tract symptoms

Introduction

Benign prostatic hyperplasia (BPH) is a major health concern that increases with the gradual aging of the population (Roehrborn, 2011). In BPH, smooth muscle and epithelial cells grow primarily within the prostatic transition zone, and this pathological process induces unpleasant lower urinary tract symptoms (LUTS) (Roehrborn, 2011). Inflammation plays an important role in the development of BPH. Histological evidence of prostatic inflammation is present in approximately

90% of samples taken during transurethral resection of the prostate (TURP) (Kessler *et al.*, 1998). Evidence suggests that immune-mediated inflammatory stimuli disrupt the homeostasis between cell proliferation and cell death. As a result, proliferative processes predominate, and apoptotic processes (i.e. increased expression of the anti-apoptotic protein Bcl-2 and reduced activity of the pro-apoptotic proteins BAX and caspase-9) are inhibited in the prostate (Lee and Peehl, 2004; Kramer *et al.*, 2007; Sciarra *et al.*, 2007; 2008). Inflammation contributes to tissue injury through up-regulation of protein and lipid mediators released by inflammatory cells driving local angiogenesis and production of growth factors such as VEGF and EGF (Lucia and Lambert, 2008).

PGs are important inflammatory mediators arising from the conversion of arachidonic acid (AA) mediated by two different isoforms of COX (COX-1 and COX-2). Both COX-1 and COX-2 are expressed in the prostate gland (Kirschenbaum *et al.*, 2000). COX-2 is a pro-inflammatory, inducible enzyme whose production is triggered by mitogens, cytokines, reactive oxygen species and growth factors in different cell types. Increased expression of COX-2 mRNA has been documented in BPH, particularly in luminal epithelial cells (Kirschenbaum *et al.*, 2000). Several mechanisms have been proposed to explain the role of COX-2 in prostate overgrowth. Some of these effects may result from the COX-2-mediated increases in PG synthesis, especially of PGE_2 (Larré *et al.*, 2008) but COX-2 also up-regulates anti-apoptotic protein Bcl-2 expression with a concomitant decrease in prostate tissue apoptosis (Tsujii and DuBois, 1995).

AA is also converted to leukotriene A_4 (LTA_4), the intial component of the LT biosynthetic pathway, by 5-lipoxygenase (5-LOX). This enzyme is over-expressed in prostate cancer (Gupta *et al.*, 2001) and inhibitors of 5-LOX trigger the apoptotic machinery in prostate cancer cell lines such as LNCaP and PC3 (Ghosh and Myers, 1998). Inflammatory cells such as polymorphonuclear leukocytes and macrophages contain the LTA_4 hydrolase that transforms LTA_4 into LTB_4. LTB4 attracts leukocytes to the site of inflammation, promoting their adhesion to the inflamed and damaged tissue. Interestingly, increased levels of LTB_4 are found in BPH and may amplify the inflammatory cascade that accompanies the benign overgrowth of the prostate gland. All these experimental findings suggest that a dual inhibitor of the COX and 5-LOX enzymes might reduce BPH. To date, experimental studies have not specifically addressed this possibility.

Flavonoids from *Scutellaria baicalensis* (Chinese skullcap) and *Acacia catechu* (black catechu) are found in traditional medicines used for the treatment of several clinical conditions. Mixtures of these plant extracts have anti-inflammatory, antiviral, antibacterial, anticancer and cardiovascular effects (Van Loom, 1997; Chou *et al.*, 2003; De Clerq, 2005; Huang *et al.*, 2005). Flavocoxid contains both baicalin and catechin, which inhibit *in vitro* the peroxidase activities of COX-1 and COX-2, rather than the cyclooxygenase activity of these enzymes, and the 5-LOX enzyme (Burnett *et al.*, 2011). *In vitro*, flavocoxid also reduces COX-2 and 5-LOX mRNA expression and affects gene expression as well as protein levels of inflammatory markers, such as TNF-α, from immune-inflammatory cells (Altavilla *et al.*, 2009). *In vivo* studies have confirmed the strong anti-inflammatory activity of flavocoxid. Flavocoxid reduced the severity of experimental pancreatitis (Polito *et al.*, 2010) and the exaggerated muscle necrosis and inflammation in *mdx* mice, an experimental model of Duchene dystrophy disease (Messina *et al.*, 2009).

In this study, the efficacy of flavocoxid in experimental BPH was investigated using both *in vivo* and *in vitro* approaches to assess the therapeutic potential of this dual inhibitor. Both models of prostatic inflammatory disease may be important to validate the efficacy of a broad spectrum anti-inflammatory drug.

Methods

In vivo *experiment*

Animals. All animal care and experimental 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) and were approved by the Ethics Committee of the University of Messina. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 28 male Sprague–Dawley rats (3 months old, weighing 220–250 g) were purchased from Charles River Laboratories (Calco, Milan, Italy). Animals were maintained in plastic cages under standard environmental conditions with water and food *ad libitum* at the Animal Facility of the Department of Clinical and Experimental Medicine and Pharmacology of the University of Messina, Messina, Italy.

After 1 week of acclimation to the laboratory environment, animals were randomly assigned to four groups: Sham-BPH treated with vehicle ($n = 7$; 100 µL corn oil s.c.); sham-BPH + flavocoxid ($n = 7$; 20 mg kg^{-1}, i.p.); BPH ($n = 7$; testosterone propionate 3 mg·kg^{-1}, s.c. diluted in corn oil in a volume of 100 µL) and BPH + flavocoxid treated with testosterone and flavocoxid ($n = 7$; 20 mg·kg^{-1}, i.p.) for 14 days. At the end of the experiment, animals were killed under ether anaesthesia, and their prostates were immediately removed and used for further analysis. For flavocoxid (Primus Pharmaceuticals, Inc., Scottsdale, AZ, USA) and testosterone propionate (Sigma-Aldrich, Milan, Italy), the doses and routes of administration were chosen according to previously published reports (Pandita *et al.*, 1998; Arruzazabala *et al.*, 2004; Messina *et al.*, 2009; Polito *et al.*, 2010).

Prostate weight. At study completion, prostates were removed and weighed. Percentage of growth inhibition was calculated as follows: 100 – [(TG (treated group) – Sham) / (BPH – Sham) × 100], where TG were the values of the treated groups.

Histology. Prostates were routinely processed and embedded in paraffin, and 5 µm-thick sections were cut and stained with haematoxylin–eosin. The definitions of pathological findings of the acinar structure were based on the presence of the following: (a) villamentous or villous projections represent uniform epithelial infoldings into the lumen showing a connective tissue fine core along their longitudinal axis and are lined by epithelial cells; (b) papillary projections are

expanded epithelial infoldings with a varying number of ramifications offering a cauliflower-like pattern; (c) cribriform structure results from the fusion of contralateral papillary projections, mimicking a 'glands in glands' pattern; (d) hyperplastic nodule (a focal increase of nuclei number) displaying a multilayered conglomerate.

Real-time PCR. Total mRNA was extracted and quantified from prostate samples as previously described. Briefly, total RNA was extracted from prostate and after reverse transcription cDNA was stored at –80°C. The reversed transcribed product was used to quantify the amount of Bax, Bcl-2, VEGF and EGF by real-time PCR method. To control for overall RT-PCR activity between samples, β-actin cDNA was used as endogenous control. Target as well as control genes were assayed using specific TaqMan probes from Applied Biosystems (http://www.appliedbiosystems.com). The results from target gene experiments were expressed as an *n*-fold difference relative to the endogenous control gene (relative expression levels).

Evaluation of LTB_4 and PGE_2 in prostate tissue. Prostate samples were homogenized and assayed for LTB_4 using a commercially available ELISA kit (R&D Systems, Milan, Italy) 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 on homogenised samples without purification by using the Cayman EIA kit (Cayman, Arcore, Milan, Italy). Samples were run in duplicate, and the absorbance was spectrophotometrically read at 412 nm. Signal intensity was directly proportional to the content of PGE_2 in samples.

In vitro *experiments*

Cell treatment and viability. Human prostate cancer PC3 cells were obtained from ATCC (distributed by LGC Standards, Milan, Italy) and grown in the specific F-12 K medium (LGC Standards) supplemented with 10% fetal calf serum, 100 $U \cdot mL^{-1}$ of penicillin, 100 $ng \cdot mL^{-1}$ of streptomycin (Life Technologies, Inc., Invitrogen, Milan, Italy). To test the effects of flavocoxid on cell viability, cells were plated in 100 mm diameter dishes at a density of 5×10^5 cells·per dish, cultured in complete medium to 70% confluency then treated with vehicle (20% DMSO in F12-K medium), or different doses of flavocoxid (256, 500, 1000 $\mu g \cdot mL^{-1}$) for 24 h. Cell viability was assessed using Trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

For caspase inhibition experiments, the cells were incubated with N-benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-FMK; R&D Systems, Inc.), a general caspase inhibitor, at 10 $\mu mol \cdot L^{-1}$, for 4 h before the addition of flavocoxid.

Real-time PCR. At 24 h cells were washed with medium and adherent cells were treated with Trizol LS (Life Technologies), then total RNA were extracted following the manufacturer's procedures. RNA was then used, as described above, to quantify the amount of BAX, Bcl-2, VEGF and EGF.

Western blot analysis of COX-2, 5-LOX and apoptosis markers. Protein samples (20 µg) from PC-3 cells prepared by lysing cells in specific buffer were separated by electrophoresis, transferred onto nitrocellulose membranes and incubated with a primary antibody for COX-2, 5-LOX, total and cleaved caspase-9 (Abcam, Cambridge, UK), PARP, cleaved-PARP, annexin V, cytochrome c (Abcam), BAX or Bcl-2 (Chemicon, Temecula, CA, USA) overnight at 4°C. The next day, membranes were incubated with a specific peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) and analysed by the Enhanced Chemiluminescence system. The protein signals were quantified using a bio-image analysis system (Bio-Profil, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared with those of control normal rats measured with the same batch. β-actin (Cell Signalling, Danvers, MA, USA) was used on stripped blots to confirm equal protein loading.

Statistical analysis

Data are shown as means ± SEM unless otherwise stated. Comparison between groups was performed using the Kruskal–Wallis one-way test. Differences between individual treatment groups were compared using Dunn's test. $P < 0.05$ was considered significant. Statistical analysis was performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Materials

Testosterone propionate was obtained from Sigma-Aldrich. Flavocoxid, a mixture of > 90% purified baicalin and catechin at a ratio of approximately 4.5:1 with the remainder being excipient (5–6%) and water (3%) (Altavilla *et al.*, 2009) was a kind gift from Primus Pharmaceuticals, Inc. All substances were prepared fresh daily. Flavocoxid stock solution was prepared with 100% DMSO. All the experiments were carried out with a 10 times diluted working solution in 0.9% NaCl.

Results

Prostate weight

Table 1 shows prostate weight at the end of the experimental period. Flavocoxid (20 $mg \cdot kg^{-1}$, i.p.) did not significantly change the weights of the prostate from sham BPH rats (data not shown). Chemical stimulation with testosterone induced BPH and, after 14 days of treatment, prostate weight markedly increased (Table 1). Flavocoxid, given daily at 20 $mg \cdot kg^{-1}$, inhibited prostate enlargement and growth by 91% in BPH animals. A 10% reduction in prostate growth rate was observed in Sham animals treated with flavocoxid, suggesting a mild reduction of prostate weight.

Prostate histology

Prostate glands harvested from sham BPH rats treated with vehicle or flavocoxid showed normal histology (Table 2 and Figure 1A and B). By contrast, prostates obtained from BPH

rats treated with vehicle had a complete disorganisation of the prostate tissue and a marked hyperplasia, compared with sham BPH animals (Table 2; Figures 1C). Flavocoxid treatment of BPH rats diminished the altered histology and the hyperplasia (Table 2; Figure 1D).

Prostate COX-2 and 5-LOX expression

Prostates from sham BPH animals treated with vehicle or flavocoxid showed a low constitutive expression of COX-2 and 5-LOX (Figure 2A and B). Prostates harvested from BPH animals injected with vehicle showed a marked increase in expression of both proteins (Figure 2A and B). Treatment with flavocoxid reduced the increased expression of COX-2 and 5-LOX (Figure 2A and B).

Prostate PGE_2 and LTB_4 production

Homogenates of the prostates from sham BPH animals treated with vehicle had low, but detectable, levels of PGE_2 and LTB_4 (Figure 2C and D). Prostates harvested from BPH animals injected with vehicle showed a marked increase in PGE_2 and LTB_4 content (Figure 2C and D). Treatment with flavocoxid reduced this increase in PGE_2 and LTB_4 (Figure 2C and D).

Table 1

Effects of treatments on prostate growth in rats treated with testosterone

Group	Prostate weight (in g; mean ± SD)	Growth inhibition (%)
Sham	0.75 ± 0.03	–
Sham + Flavocoxid	0.68 ± 0.05*	9
BPH	1.53 ± 0.05	–
BPH + Flavocoxid	0.82 ± 0.05#	91#

*$P < 0.05$ significantly different from Sham. #$P < 0.001$ significantly different from BPH.

Prostate expression of Bax, Bcl-2, caspase-9, VEGF and EGF

Prostates from sham animals treated with vehicle or flavocoxid had detectable levels of Bax and Bcl-2 evaluated as mRNA and protein (Figure 3A and B; Figure 4B and C). Prostate tissue from BPH rats showed a slight increase in Bcl-2 mRNA and protein level without changes in expression of pro-apoptotic Bax (Figure 3A and B; Figure 4B and C). Administration of flavocoxid reduced the anti-apoptotic Bcl-2 gene expression and increased pro-apoptotic Bax in the prostates of BPH animals (Figure 3A and B; Figure 4B and C). Caspase-9 was evaluated as total protein and in its active form, showing an increase in the cleaved isoform as well as a parallel decrease in the total isoforms in BPH, compared with sham animals given either vehicle or flavocoxid. BPH animals receiving flavocoxid showed a further increase in active caspase-9 with a reduction in the total isoforms (Figure 4A). The ratio between total and the cleaved caspase-9, in favour of the latter, strongly suggests that flavocoxid mediates a caspase-dependent activation of apoptosis.

Prostate glands harvested from sham animals injected with vehicle or flavocoxid showed also measureable levels of mRNA for the growth factors VEGF and EGF (Figure 3D and E). Both EGF and VEGF mRNA levels were significantly enhanced in the prostates from BPH rats (Figure 3D and E). Flavocoxid treatment significantly reduced the expression of both growth factors in BPH prostates (Figure 3D and E).

In vitro *experiments*

In cultures of PC3 prostate cancer cells, viability and cell number were markedly reduced (Figure 5A and B), as determined by the MTT assay, after 24 h of incubation with flavocoxid. The most effective dose of flavocoxid in reducing cell viability was 500 $\mu g \cdot mL^{-1}$. These results were confirmed under light microscopy, with the hormone-resistant prostate cancer PC3 cells showing a significant reduction when they were cultured for 24 h with flavocoxid. In agreement with this finding, Bax and caspase-9 were significantly up-regulated in the flavocoxid-treated cells compared with non-treated con-

Table 2

Histopathological features shown in Sham, Sham + Flavocoxid, BPH and in BPH + Flavocoxid rats

	Sham	Sham + Flavocoxid	BPH	BPH + Flavocoxid
Acinar regularity	Present	Present	Absent	Present
Stroma	Slight	Slight	Variable	Slight
Cell shape	Cuboidal/low cylindrical	Cuboidal/low cylindrical	Cylindrical	Cuboidal/low cylindrical
Cell polarity	Present	Present	Present/absent	Present
Nuclear shape	Round	Round	Round/ovoid	Round/ovoid
Mitoses	Absent	Absent	Isolated	Absent
Acinar villi	Absent	Absent	Slight	Slight (focally)
Cribiform pattern	Absent	Absent	Absent	Absent
Piling-up	Absent	Absent	Moderate	Absent
Budding-out	Absent	Absent	Slight	Absent
Basal membrane	Intact	Intact	Intact/Focal interruption	Intact

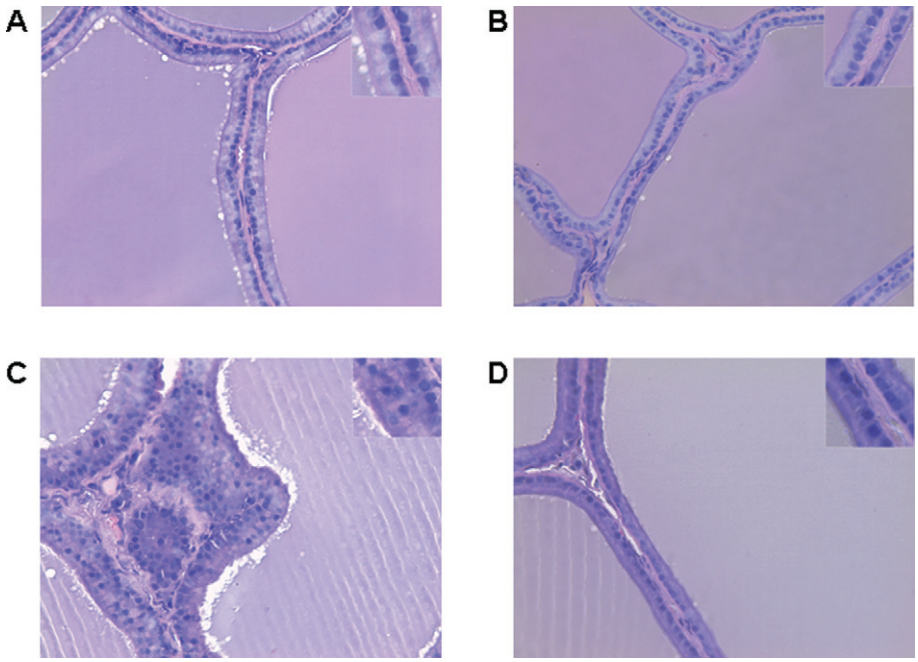

Figure 1

A. Sham prostate from vehicle-treated rats showing regular acini with cuboidal and low cylindrical epithelium with round nuclei showing basal alignment. Fine stroma and a continuous basal layer are also noted. Haematoxylin and eosin staining; original magnification, ×20. B. Sham prostate from flavocoxid-treated rats showing regular architecture comparable to sham animals treated with vehicle. Haematoxylin and eosin staining; original magnification, ×20. C. BPH prostate showing irregular acinar shape with papillary projection into the lumen and foci of piling-up hyperplastic nodules are evident. The epithelium is high cylindrical, multilayered and round/ovoid nuclei are irregularly aligned. There are abundant stroma and a focal interruption of basal layer surrounding the acini. Haematoxylin and eosin staining; original magnification, ×20. D. BPH prostate treated with flavocoxid displaying histological features of normal prostate structure. Haematoxylin and eosin staining; original magnification, ×20.

trols, while the anti-apoptotic Bcl-2 gene showed a decreased expression after flavocoxid treatment compared to the control (Figure 6). The evaluation of the expression of Annexin V, PARP and cleaved-PARP as well as the cytosolic release of cytochrome c revealed a significant induction of apoptosis in flavocoxid treated cells (Figure 7). Incubation with the pan-caspase inhibitor, Z-VAD-FMK, countered flavocoxid-induced, caspase-related apoptosis, as demonstrated in Figures 6 and 7. Finally, the expression of the growth factors EGF and VEGF (Figure 8) was markedly inhibited by incubation with flavocoxid.

Discussion

BPH is the most common benign tumour in men, showing an age-related incidence (Donnell, 2011). BPH represents a pattern of unregulated but non-malignant growth characterized by an increase in prostate epithelial and stromal cells (Donnell, 2011). The exact pathogenesis of BPH is still unclear, but several clinical and experimental observations indicate that inflammation in the prostate gland is closely associated with the development of BPH (Fibbi *et al.*, 2010).

Of the several inflammatory pathways, aberrant AA metabolism (i.e. elevated COX-2 and 5-LOX expression and increased levels of PGE_2 and LTB_4) has been linked with the development of BPH (Kirschenbaum *et al.*, 2000; Larré *et al.* 2008). If both COX-2 and 5-LOX over-expression is associated with BPH, dual inhibitors of these two key enzymes, at both the AA metabolic and the genomic level, should be investigated as new targets for the pharmacological treatment of BPH. Indeed, previous findings indicated that two COX-2 selective inhibitors, rofecoxib and celecoxib, are effective in the management of LUTS in human BPH either as a combination therapy with finasteride or as monotherapy respectively (Di Silverio *et al.*, 2005; Falahatkar *et al.*, 2008). Because of their side-effect profile and possible comorbidities in renal and cardiovascular function, the NSAIDs, including selective COX-2 inhibitors, are not advised for a chronic condition such as BPH. Flavocoxid has indeed demonstrated less toxicity compared to NSAIDs in recent trials, mainly due to its balanced inhibition of COX and LOX.

Finasteride is approved for the treatment of BPH, as a 5-α-reductase inhibitor, which prevents the conversion of testosterone to dihydrotestosterone. In PC3 cells, finasteride induced apoptosis through increased Bax, decreased Bcl-2 and up-regulated caspase-3 activity (Golbano *et al.*, 2008). The associated side effect profile of finasteride treatment (>2%) includes impotence (5–8%), decreased libido (3–6%), abnormal ejaculation and decreased ejaculatory volume (2–5%), abnormal sexual function (2.5%) and breast enlargement (0.5–2%) (Merck & Co., Inc., Whitehouse Station, NJ, USA). There is also a low incidence of testicular pain but, more seriously, a measurable incidence of high-grade prostate cancer (FDA

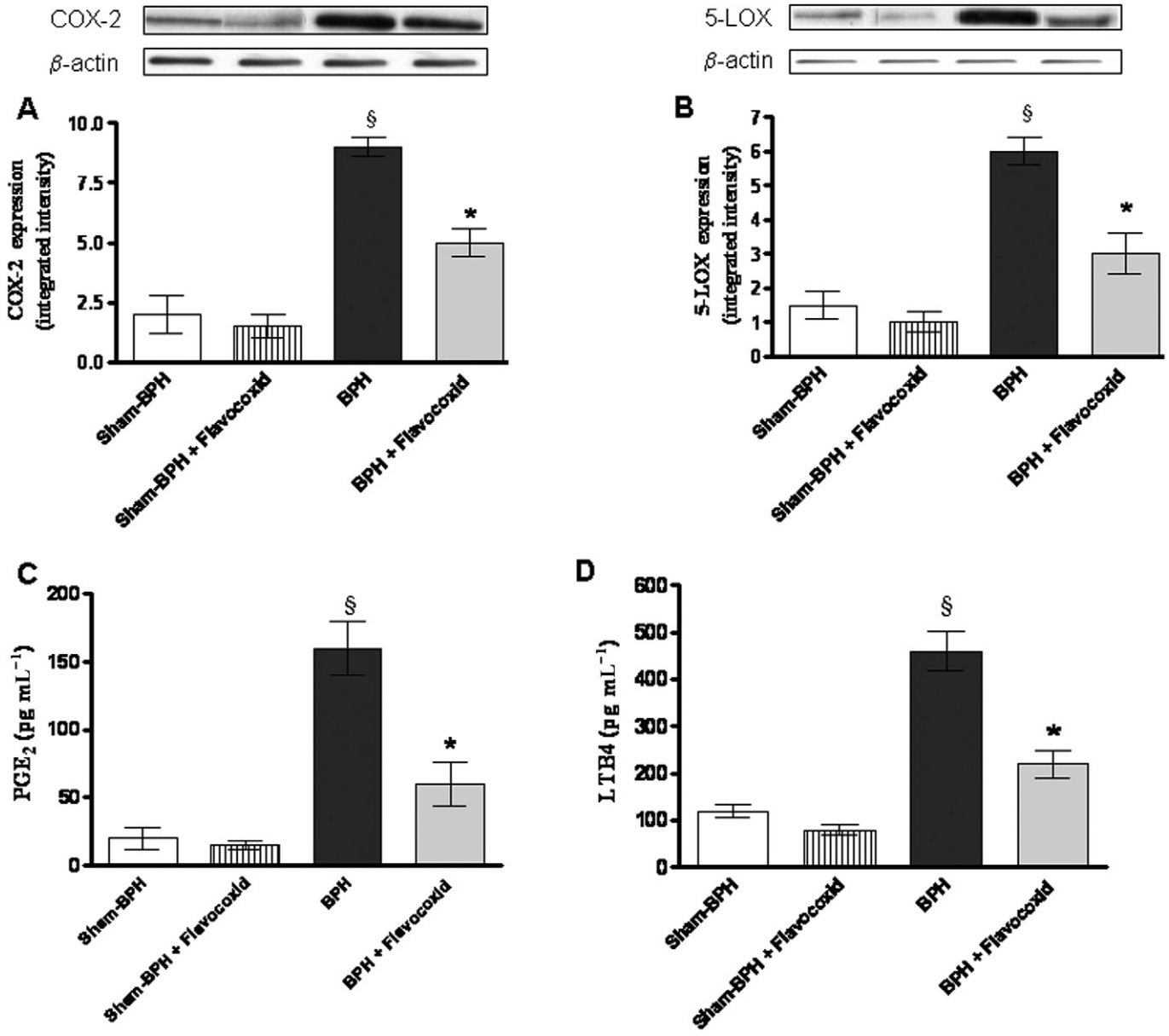

Figure 2

Western blot analysis of COX-2 (A) and 5-LOX (B) in prostate from sham BPH and BPH rats treated with either vehicle or flavocoxid. PGE_2 (C) and LTB_4 (D) levels in homogenates of samples of prostate from sham BPH and BPH rats treated with either vehicle or flavocoxid. §$P < 0.01$ significantly different from Sham-BPH; $P < 0.05$ significantly different from BPH.

Safety Notice, 2011). These known side effects represent a risk for men with a chronic condition. A safe therapeutic option is therefore still needed for BPH, which could manage the symptoms of the enlarged prostate without major effects on other organs, chance of sexual dysfunction or risk of cancer.

BPH arises from an imbalance between cell proliferation and cell death. Inflammation, mediated by elevated COX-2 and 5-LOX enzyme activity and accumulation of their main AA metabolic end-products, PGE_2 and LTB_4, may have an important role in perturbing cell homeostasis. Indeed, COX-2 and PGE_2 are known to induce inhibition of apoptosis via an increased expression of the anti-death protein Bcl-2 and to stimulate the production of several growth factors (Sheng *et al.*, 1998; Majima *et al.*, 2000; Nakanishi *et al.*, 2001; Sumitani *et al.*, 2001). Similar effects have been also described for 5-LOX and LTB_4. Both, in fact, inhibit the apoptotic machinery and promote cell proliferation and growth (Nieves and Moreno, 2006; Wada *et al.*, 2006; Tong *et al.*, 2007; Zhou *et al.*, 2007). There is evidence that indicates that a combined inhibition of COX-2 and 5-LOX leads to a greater reduction of prostatic cell growth compared with inhibiting each enzyme individually (Schroeder *et al.*, 2007).

Among the COX-2 inhibitors, only celecoxib induces apoptosis of PC3 cancer cells (Andrews *et al.*, 2008), whereas

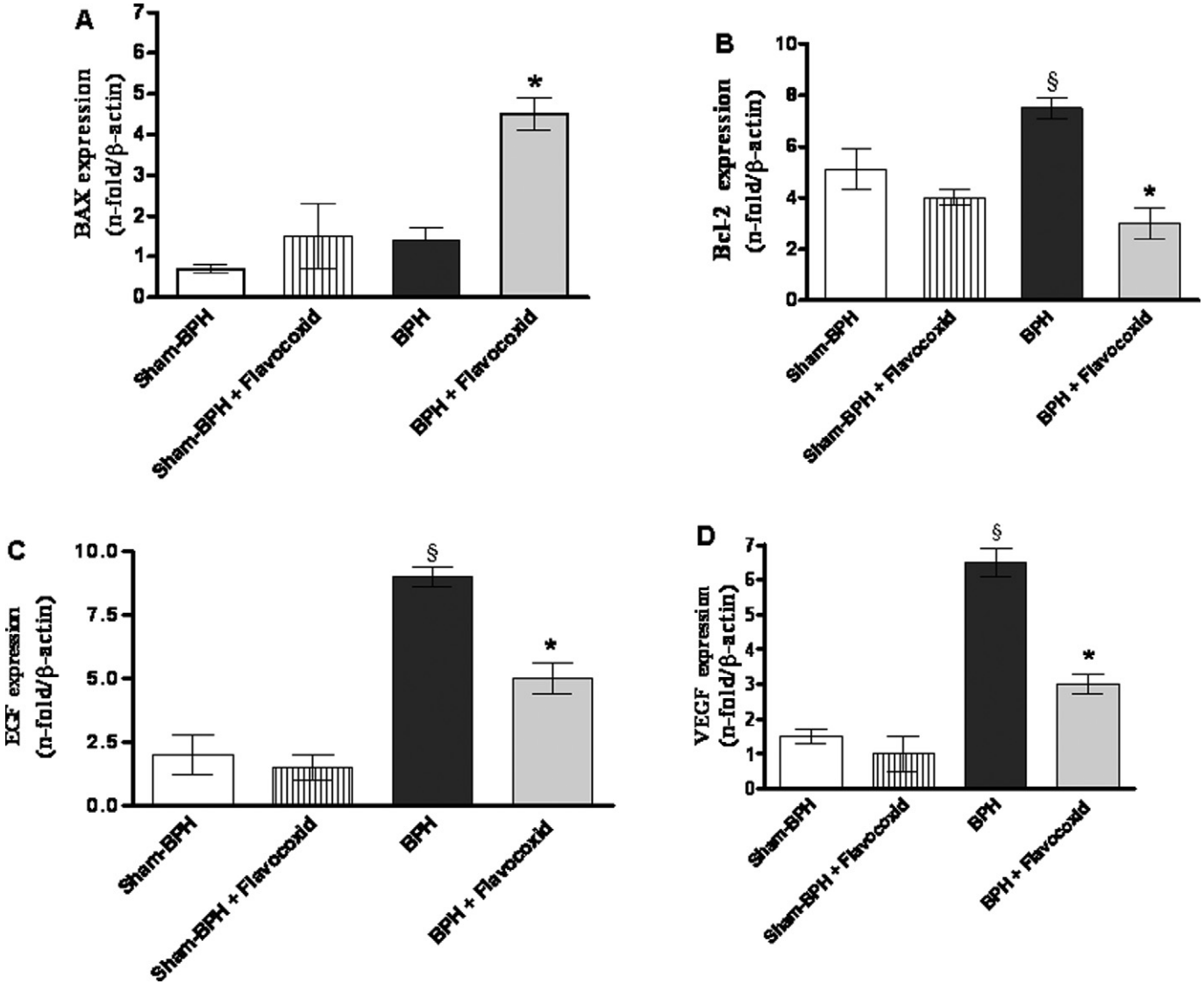

Figure 3

Expression of mRNA for Bax (A), Bcl-2 (B), EGF (C) and VEGF (D) in samples of prostate from sham-BPH and BPH rats treated with either vehicle or flavocoxid. §$P < 0.01$ significantly different from Sham-BPH; *$P < 0.05$ significantly different from BPH.

Barry *et al*. (2009) found that dual inhibition of COX and 5-LOX decreased growth of human breast cancer cells. There are several reports of the effect of the combined action of NSAIDs (that inhibit COX enzymes) and the inhibitors of 5-LOX in different cell types, conditions and diseases. The topical application of zileuton, a 5-LOX inhibitor, and celecoxib reduced the incidence of skin cancer (Fegn and Wang, 2009). This anticancer activity, however, may be due to a mechanism other than inhibition of LT production. In 5-LOX-positive Capan-2 pancreatic cancer cells, zileuton failed to show an anti-proliferative effect, whereas other 5-LOX inhibitors were anti-proliferative and some of them induced cytotoxicity (Fischer *et al*., 2010). In other cancer-derived cell lines, COX-2 and 5-LOX inhibition suppressed the growth, compared with zileuton alone (Chen *et al*., 2004; Ye *et al*., 2005), decreasing Bcl-2 expression and increasing Bax expression (Cianchi *et al*., 2006). Indomethacin plus nordihydroguaiaretic acid, a 5-LOX inhibitor, down-regulated apoptosis in macrophages induced by AA (Muralidhar *et al*., 2004). Similarly, dual inhibitors of COX and 5-LOX inhibit cell growth. Narayanan *et al*. (2007) reported licofelone, a dual inhibitor of COX-2 and 5-LOX, prevented overall cancer cell growth by enhancing apoptosis in both androgen-dependent and androgen-independent PC cells. In HCA-7 colon cancer cells, licofelone induced Bax in the apoptotic process and blocked the activity of 5-LOX and COX enzymes (Tavolari *et al*., 2008). Other mechanisms may also play a role in the action of specific agents used to treat BPH.

There is growing evidence for the involvement of oxidative stress in BPH (Pace *et al*., 2010). Specific markers of oxidative stress have been implicated in BPH, along with genetic changes that make individuals more susceptible to damage

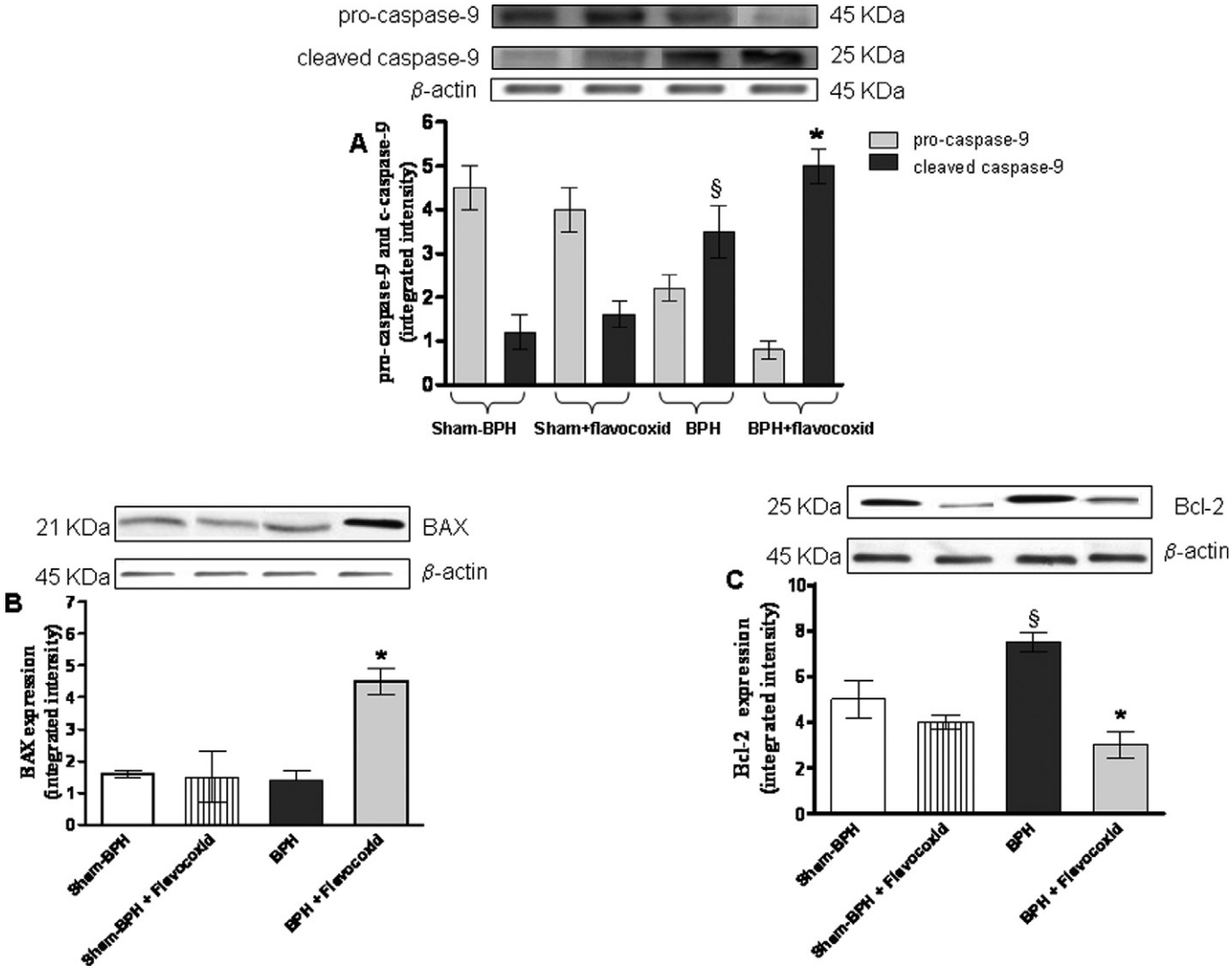

Figure 4

Western blot analysis for caspase-9 (A), BAX (B) and Bcl-2 (C) in samples of prostate from sham BPH and BPH rats treated with either vehicle or flavocoxid. §$P < 0.01$ significantly different from Sham-BPH; $P < 0.05$ significantly different from BPH.

from specific reactive oxygen species (Kedia *et al.*, 2008; Kumar *et al.*, 2011). Several plant extracts and phytochemicals, with high antioxidant capacity, inhibit pathways that up-regulate cell growth and promote management of BPH (Khan *et al.*, 2009; van Breemen *et al.*, 2011; Larré *et al.*, 2011). Polyphenol compounds or mixtures of flavonoids, such as flavocoxid, may represent a therapeutic approach to the safe management of BPH, through the combined action of antioxidant effects as well as COX and 5-LOX inhibition.

Flavocoxid, a mixture of purified baicalin and catechin at a ratio of approximately 4.5:1 (>90%), 5–6% excipient and water at ~3% (Altavilla *et al.*, 2009), inhibits the peroxidase activity of the COX enzymes, rather than their cyclooxygenase activity, the target of the NSAIDs (Burnett *et al.*, 2011). In addition, flavocoxid has weak inhibitory activities towards cytoplasmic phospholipase A2 ($cPLA_2$) and 5-LOX along with a strong and broad antioxidant activity to modulate inducible gene expression. Altavilla *et al.* (2009) showed that this broad anti-inflammatory activity depressed PGE_2 and LTB_4 production and also modulated NF-κB, increased IκBα expression, decreased TNF-α and acted as general antioxidant preventing the generation of malondialdehyde from AA via a peroxide radical.. Mixtures of baicalin and catechin have also been shown to decrease COX-2 gene expression (Tseng-Crank *et al.*, 2010), whereas celecoxib and ibuprofen up-regulated mRNA expression of COX-2 (Burnett *et al.*, 2011). In the present study, treatment with flavocoxid significantly inhibited PC3 cell growth as well as the prostate enlargement *in vivo*, probably through both a caspase-dependent signal, involving caspase-9, and another, separate, mechanism involving the pro-apoptotic Bax and the anti-apoptotic Bcl-2 gene.

To date, there are three major apoptotic signalling pathways that have been described: the mitochondrial pathway, the endoplasmic reticulum pathway and the death receptor pathway (Daniel, 2000). Bcl-2 and Bax are key players in

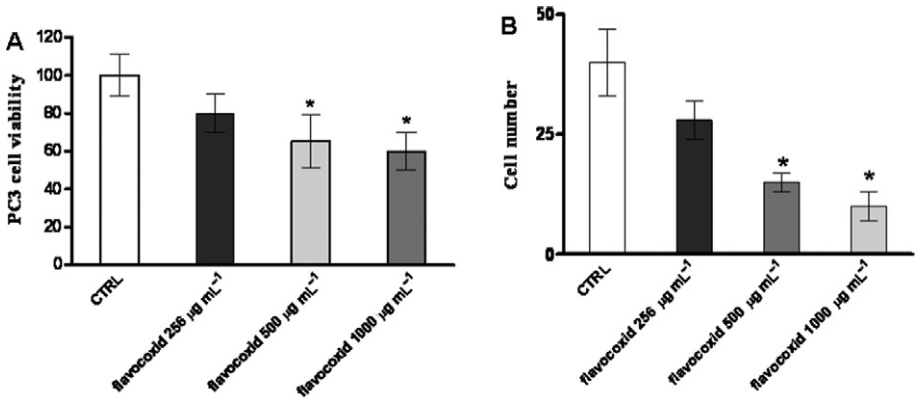

Figure 5

Effect of incubation with flavocoxid (256-1000 μg mL^{-1}) on PC3 cell cultures. The graphs represent cell viability (A) and cell growth, as number of live cells in each field (B). *$P < 0.01$ significantly different from CTRL.

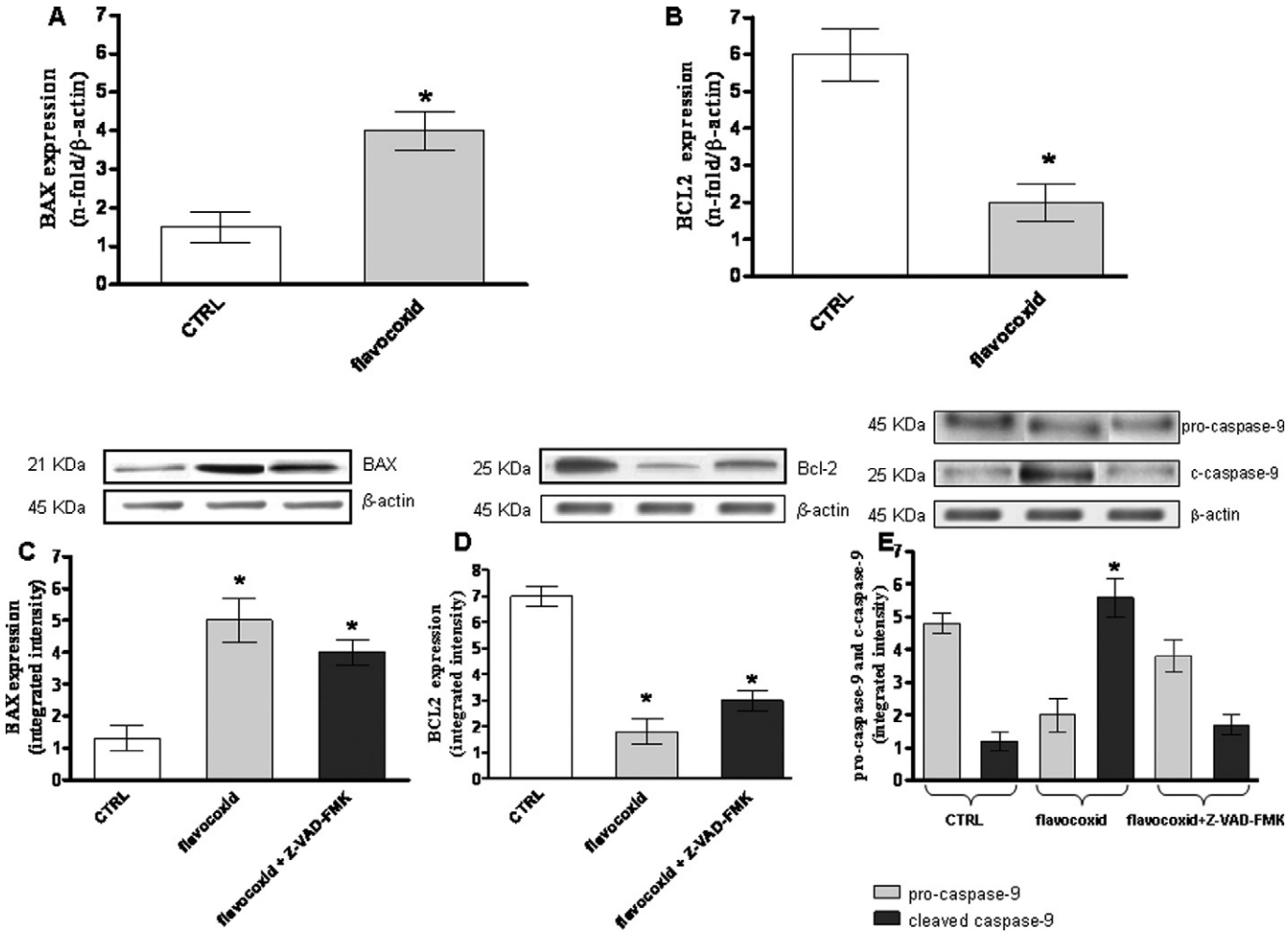

Figure 6

Bax (A), Bcl-2 (B), mRNA and BAX, Bcl-2 and caspase-9 protein (C, D and E, respectively) expression in PC3 cells treated with vehicle, flavocoxid (500 μg·mL^{-1}) or the pan-caspase inhibitor Z-VAD-FMK and flavocoxid. *$P < 0.01$ significantly different from CTRL.

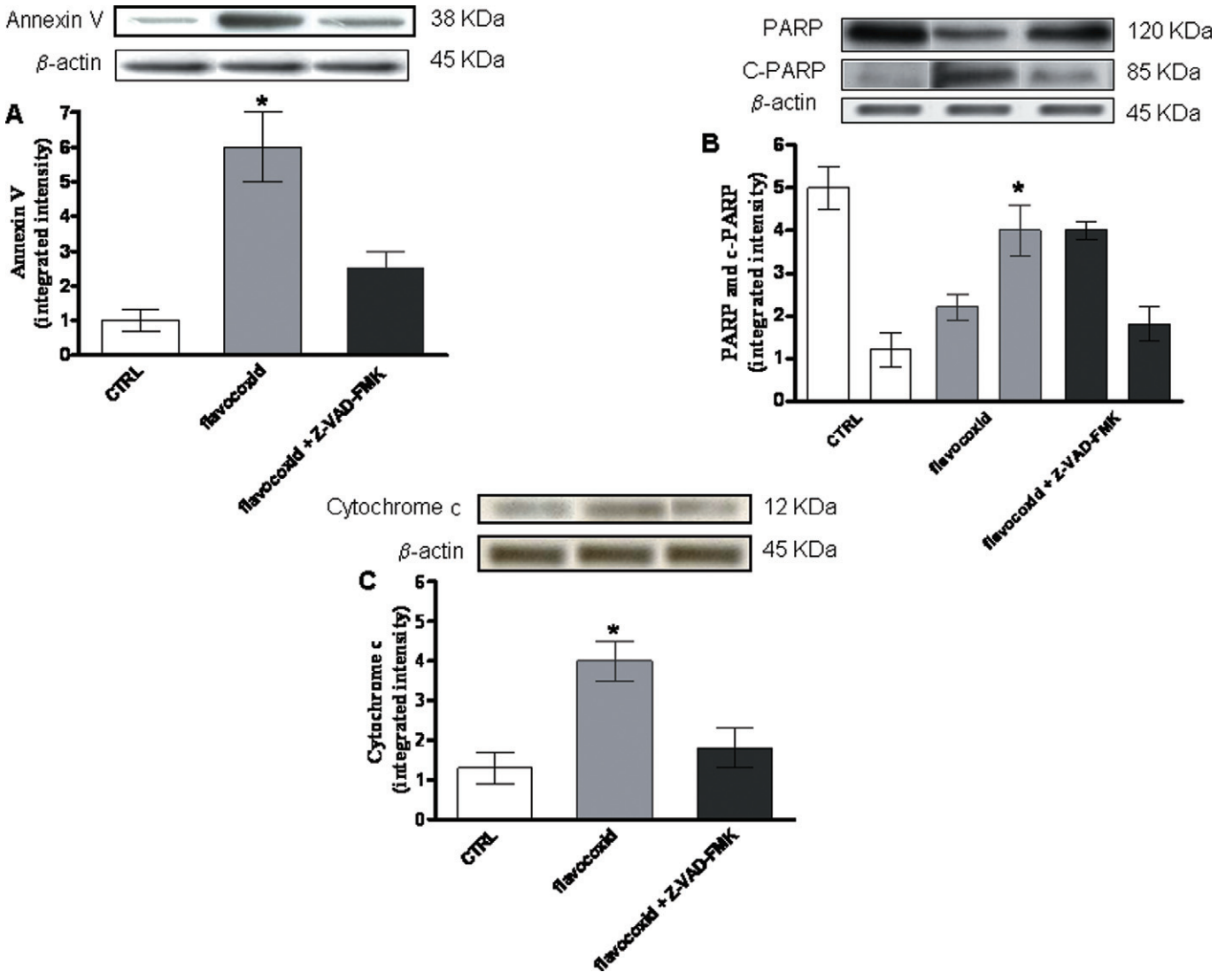

Figure 7

Annexin V (A), PARP (B) and cytochrome c protein (C) expression in PC3 cells treated with vehicle, flavocoxid (500 μg ml^{-1}) or the pan-caspase inhibitor Z-VAD-FMK and flavocoxid. *$P < 0.01$ significantly different from CTRL.

apoptotic events (Figure 9). Bcl-2 is an upstream effector molecule in the apoptotic pathway, and it has been identified as a potent suppressor of apoptosis (Hockenbery *et al.*, 1993). Most cancers including prostate cancer generally over-express Bcl-2 (Reed, 1995; Revelos *et al.*, 2005), thereby escaping apoptosis and reducing therapeutic efficiency. Bcl-2 forms a heterodimer with the apoptotic protein Bax neutralizing its apoptotic effects. The ratio of these proteins determines whether a cell will undergo apoptosis. Here we showed that flavocoxid significantly reduced Bcl-2 protein and increased levels of Bax during BPH. The endoplasmic reticulum caspase-driven cascades can be activated by multiple pathways, including a membrane death receptor pathway that triggers activation of the initiator caspase-8 or through the mitochondrial pathway, which relies on cytochrome c release and activation of the initiator caspase-9 (Green and Reed, 1998; Kim, 2002). These data suggest that BPH causes a pro-apoptotic event by increasing cleavage of caspase-9, and that the cells respond to this injury by up-regulating Bcl-2 in a compensatory response. Interestingly, flavocoxid seems to favour apoptosis by increasing the caspase-9 signalling further but also by inhibiting Bcl-2 expression, which causes an increase in BAX expression. This mechanism differs from other anti-inflammatory agents.

Celecoxib exhibits a number of activities apart from inhibiting COX-2. This it also inhibits NF-κB and PPARγ,; arrests cell cycle at the G1–S phase transition point in a p53-dependent manner; decreases a number of cell cycle regulatory proteins; and induces apoptosis activating factor-1, and caspase-3 (Narayanan *et al.*, 2003; Han *et al.*, 2004.; Dvory-Sobol *et al.*, 2006; Kang *et al.*, 2009). Interestingly, baicalein, a derivative produced by gut flora from the baicalin in flavocoxid, itself decreased PC cell proliferation by arresting the G0/G1 phase and was associated with suppres-

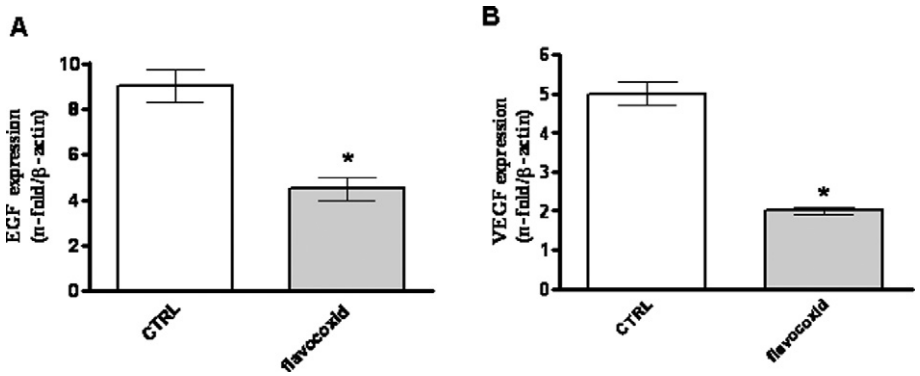

Figure 8

Expression of mRNA for EGF (A) and VEGF (B) in PC3 cells treated with vehicle or flavocoxid (500 μg·mL^{-1}) *P < 0.01 significantly different from CTRL

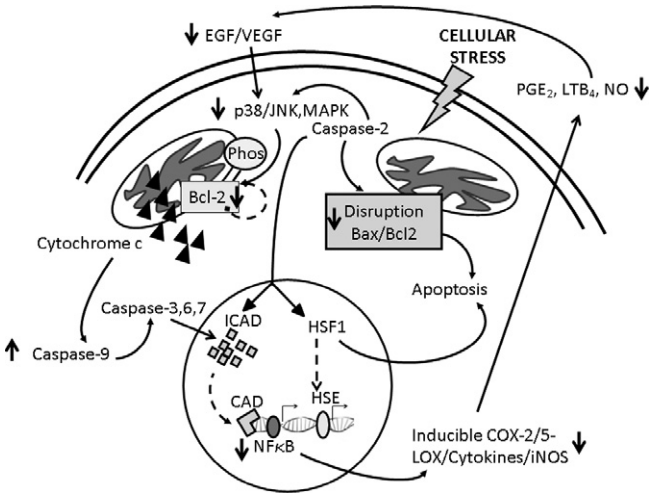

Figure 9

The effect of flavocoxid on specific markers of apoptosis is shown by ↓ (decrease) and ↑ (increase). Inducible NOS (iNOS); inhibitor of caspase-3-activated DNase (ICAD) is a caspase-3 substrate; caspase-3-activated DNase; heat shock factor 1 (HSF1); heat shock element (HSE); Bcl-2–associated X protein(Bax); B-cell lymphoma 2(Bcl-2).

sion of cyclin D1 and D3 protein levels (Pidgeon *et al.*, 2002). Baicalin, the glycoside of baicalein, is a specific 5-LOX inhibitor (Burnett *et al.*, 2007) but works with catechin as a peroxidase inhibitor on the COX enzymes as well (Burnett *et al.*, 2011). In this study, flavocoxid induced over-expression of caspase-9 in prostate glands, further stimulating the apoptotic machinery triggered by the Bax/Bcl-2 imbalance.

MK591, a specific inhibitor of 5-LOX LT formation, induces apoptosis in prostate cancer cells in an Akt- and ERK-independent manner (Sarveswaran *et al.*, 2011). Nordihydroguaiaretic acid in combination with TNF-related apoptosis-inducing ligands also induced apoptosis (Yoshida *et al.*, 2007). This induction of apoptosis, however, did not involve different levels of expression for Bcl and other apoptotic factors. A caffeic acid phenethyl ester, a modified 5-LOX inhibitor, induced apoptosis in PC cells via NF-κB inhibition and down-regulation of inhibitors of apoptotic proteins in a caspase-dependent manner (McEleny *et al.*, 2004). It is clear from these varied activities on apoptosis in PC cells that 5-LOX inhibitors work through individual and different mechanisms to achieve cell death and may be more effective than COX inhibitors alone (Matsuyama and Yoshimura, 2008). The combined effect of 5-LOX and COX inhibitors on prostate cell growth may be additive. Not all LOX inhibitors, however, reduce the viability of prostate cancer lines (Matsuyama *et al.*, 2004a,b).

Many flavonoid molecules have been found to have different mechanisms with regard to apoptosis. Curcumin, for example, induces apoptosis through damage to mitochondrial DNA and accumulation of ceramide in cells (Hilchie *et al.*, 2010). The apoptotic process in this case is mediated by apoptosis-inducing proteins other than caspase-independent related mechanisms. Apigenin acts to disrupt the G2/M phase of the cell cycle (Wang *et al.*, 2000). Genistein, biochanin-A and apigenin were found to act via polo-like kinases and the p21-related pathway to induce apoptosis (Seo *et al.*, 2011). Therefore, each flavonoid or classes of flavonoids may act through slightly different mechanisms to induce apoptosis in cancer cells.

Inflammatory cells release cytokines as well as growth factors in order to modulate the immune response. Existing evidence shows that these growth factors promote growth of epithelial and stromal prostate cells. Growth factors play a significant role in the regulation and growth of normal, hyperplastic and malignant prostatic epithelium. Moreover, prostate cells by themselves are able to secrete inflammatory mediators and stimulate their own growth. Once this cycle begins, feedback controls of cell growth can be overwhelmed and prostate volume progressively increases. We found that during BPH there was an over-expression of the growth factor EGF, prevented by treatment with flavocoxid. EGF plays a critical role during tumorigenesis of the prostate gland (Itoh *et al.*, 1998). EGF activates intracellular signalling cascades to initiate the activation of downstream pathways, which lead to cell proliferation, migration, adhesion, anti-apoptosis, angiogenesis and metastasis (Prenzel *et al.*, 2001). EGF and its receptor EGFR are frequently over-expressed in those prostate cancers associated with a more aggressive clinical outcome

(Di Lorenzo *et al.*, 2002). Moreover, inhibition of EGFR has been shown to result in a marked decrease in Bcl-2 and a marked increase in the expression of Bax (Xiao *et al.*, 2007).

Among other growth factors involved in BPH and cancer development, VEGF stimulates neovascularization. VEGF is often called vascular permeability factor as it also enhances vascular leakage, an effect that contributes significantly to tumour development and metastasis (Bates and Curry, 1996). VEGF plays a significant role in angiogenesis and tumour growth, and BPH stromal cells, as well as prostate cancer epithelial cells, secrete large amounts of this growth factor (Jackson *et al.*, 1997). For this reason, VEGF has recently been indicated as possible therapeutic target to reduce prostate growth (Ortholan *et al.*, 2010). In our experimental models, we demonstrated a sustained anti-VEGF effect of flavocoxid.

In summary, as illustrated in Figure 9, we have demonstrated that a dual inhibition of COX-2 and 5-LOX by flavocoxid was effective in inducing apoptosis, which probably occurred through both activation of caspase-9 and Bax as well as a consequent inhibition of the Bcl-2 pathway in experimental testosterone-induced prostate hyperplasia and in hormone-independent PC3 cells. Additional anti-apoptotic effects may also be produced by flavocoxid inhibiting NF-κB-induced inflammation in terms of cytokine, COX-2 and 5-LOX expression, as well as through suppression of the p38 and JNK1 inflammatory pathways (Altavilla *et al.*, 2009; Messina *et al.*, 2009; Polito *et al.*, 2010). Flavocoxid also efficiently inhibited prostate enlargement by decreasing growth factor expression, indicating a clear therapeutic efficacy in preventing BPH. The effects of flavocoxid in PC3 cells might also suggest an anti-neoplastic role for this combination of natural compounds. This work provides support for the use of flavocoxid in initial clinical studies of BPH in humans.

Conflicts of interest

BPB is an employee of Primus Pharmaceuticals Inc., manufacturers of flavocoxid. AB and FS are consultants to Primus Pharmaceuticals, Inc.

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