



Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF- κ B activation via the NIK/IKK signalling complex

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Colorectal cancer is a major cause of cancer deaths in Western countries, but epidemiological data suggest that dietary modification might reduce these by as much as 90%. Cyclo-oxygenase 2 (COX2), an inducible isoform of prostaglandin H synthase, which mediates prostaglandin synthesis during inflammation, and which is selectively overexpressed in colon tumours, is thought to play an important role in colon carcinogenesis. Curcumin, a constituent of turmeric, possesses potent anti-inflammatory activity and prevents colon cancer in animal models. However, its mechanism of action is not fully understood. We found that in human colon epithelial cells, curcumin inhibits COX2 induction by the colon tumour promoters, tumour necrosis factor α or fecapentaene-12. Induction of COX2 by inflammatory cytokines or hypoxia-induced oxidative stress can be mediated by nuclear factor kappa B (NF- κ B). Since curcumin inhibits NF- κ B activation, we examined whether its chemopreventive activity is related to modulation of the signalling pathway which regulates the stability of the NF- κ B-sequestering protein, I κ B. Recently components of this pathway, NF- κ B-inducing kinase and I κ B kinases, IKK α and β , which phosphorylate I κ B to release NF- κ B, have been characterised. Curcumin prevents phosphorylation of I κ B by inhibiting the activity of the IKKs. This property, together with a long history of consumption without adverse health effects, makes curcumin an important candidate for consideration in colon cancer prevention.

Keywords: colon cancer; cyclo-oxygenase 2; curcumin, NF- κ B

Introduction

Cyclo-oxygenase 2 (COX2), an inducible isoform of prostaglandin H synthase (PGHS) which mediates prostaglandin synthesis during inflammation, is selectively overexpressed in colon tumours and is thought to play an important role in colon carcinogenesis (Kargman *et al.*, 1995). Genetic knock-out or pharmacological inhibition of COX2 has been shown to protect against development of colonic tumours in

mice which harbour a germline knock-out mutation of the adenomatous polyposis coli (APC) tumour suppressor gene, or in rats exposed to the colon carcinogen azoxymethane (Oshima *et al.*, 1996; Kawamori *et al.*, 1998). Germline and somatic mutations in the APC gene are important early events in human colon carcinogenesis (Nishisho *et al.*, 1991; Powell *et al.*, 1992). Overexpression of COX2 in colonic epithelial cells may promote tumour development by causing resistance to apoptosis and facilitating alterations in cell adhesion properties (Tsujii and Dubois, 1995). Non-steroidal anti-inflammatory agents (NSAIDs), which directly inhibit COX2 activity (Vane and Botting, 1995), cause regression of adenomatous polyps in familial adenomatous polyposis (FAP) patients (Giardiello *et al.*, 1995), and may reduce the risk for sporadic colon cancer (Rosenberg *et al.*, 1991; Thun *et al.*, 1991). It has therefore been suggested that COX2 is an important target for the chemopreventive effects of these agents (Dubois and Smalley, 1996; Gardiello *et al.*, 1997). However, the chronic administration of NSAIDs causes serious side-effects, thought to be due to concomitant inhibition of COX1 (Eberhart and DuBois, 1995), a constitutively expressed isoform of PGHS, making the development of selective COX2 inhibitors highly desirable. Such agents could act either by direct inhibition of the cyclo-oxygenase or peroxidase component of COX2 and/or by inhibition of COX2 gene expression (Subbaramaiah *et al.*, 1997).

Curcumin, like NSAIDs, is a potent anti-inflammatory agent due to inhibition of prostaglandin synthesis (Huang *et al.*, 1992). However, it has been shown to be only a very weak direct inhibitor of cyclo-oxygenase enzyme activity (Srivastava and Srimal, 1985). It also has chemopreventive activity in animal models of colon cancer (Rao *et al.*, 1993; Pereira *et al.*, 1996), but its mechanism of action is not well understood. It has been shown to inhibit COX2 expression (Kelley *et al.*, 1996), and in separate studies to be a potent inhibitor of NF- κ B activation (Sanjaya and Aggarwal, 1995; Bierhaus *et al.*, 1997; Kumar *et al.*, 1998), but not of its binding to DNA (Bierhaus *et al.*, 1997).

To investigate the mechanisms of colon cancer chemoprevention by curcumin we tested the hypothesis that it acts through inhibition of COX2 gene induction by the model colon tumour promoters, tumour necrosis factor α (TNF α) and fecapentaene-12. The luminal concentration of TNF α is increased in patients with inflammatory bowel disease (Braegger *et al.*, 1992), a condition which predisposes to colorectal

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cancer (Gyde *et al.*, 1998). Fecapentaene-12, the most abundant form of a group of mutagenic chemicals found in the faeces of individuals who consume a Western diet high in fat and meat (Schiffman *et al.*, 1989), has been shown to be a tumour promoter in a rat model of colorectal cancer (Zarcovic *et al.*, 1993).

The overexpression of COX2 in colon tumour cells is thought to be due to alterations in transcriptional control (Kutchera *et al.*, 1996). Several transcription factors have been implicated, but their precise roles have yet to be elucidated. The *COX2* gene is induced by a wide variety of stimuli including oncogenic viruses, growth factors, cytokines, and tumour promoters (Hershman, 1994). Overexpression in response to the viral oncogene *v-src* is mediated in part by the ras-MAP kinase signalling pathway via an AP1 transcription factor which binds to a cyclic AMP response element (CRE) in the human gene promoter (Xie and Herschman, 1995). However, overexpression caused by hypoxia in human umbilical vein endothelial cells and by IL-1 in rheumatoid synoviocytes has been shown to be mediated by NF- κ B (Crofford *et al.*, 1997; Schmedtje *et al.*, 1997). We investigated whether the chemopreventive activity of curcumin in colon cells is related to inhibition of *COX2* expression via modulation of signalling pathways that regulate the stability of the NF- κ B-sequestering protein I κ B. Serine/threonine and tyrosine kinases that mediate activation of NF- κ B by the TNF receptor 1 (TNFR1) via phosphorylation of the inhibitory protein I κ B, have recently been cloned (Malinin *et al.*, 1997; DiDonato *et al.*, 1997). They form a complex which includes NF- κ B inducing kinase (NIK) and two isoforms of the I κ B kinase (IKK α and IKK β) (Woronicz *et al.*, 1997; Cohen *et al.*, 1998). We present data showing that curcumin does indeed inhibit *COX2* expression and NF- κ B DNA binding induced by physiologically relevant concentrations of TNF α and fecapentaene-12 in human colon epithelial cells, and show that curcumin achieves this by inhibiting phosphorylation of I κ B by the NIK/IKK signalling complex.

Results

The ability of curcumin to inhibit tumour promoter-induced COX2 protein expression was first examined. Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) was the most powerful inducer of COX2 protein in human colon epithelial (HCEC) cells, causing a 25-fold induction after 6 h of treatment, which was completely inhibited by curcumin (20 μ M) (Figure 1a, lanes 2 and 3). TNF α (10 ng/ml) and fecapentaene-12 (40 μ M) caused smaller three and tenfold inductions, respectively, which were also inhibited by curcumin (Figure 1a, lanes 4–7).

We next examined the effects of TNF α (0.1–10 ng/ml) and fecapentaene-12 (1–40 μ M) on *COX2* mRNA levels by RT-PCR. TNF α caused a dose dependent induction (Figure 1b), which was maximal after 2–3 h, returning to baseline after 12 h (data not shown). Curcumin (10–40 μ M) inhibited the induction of *COX2* mRNA by TNF α , also in a dose dependent manner (Figure 1c, samples 4–6). Similar induction was evident after treatment with fecapentaene-12, which was inhibited by 75% in the presence of 40 μ M

curcumin (data not shown). The viability of cells after 4 h of treatment was greater than 90%, as determined by the MTT assay. Curcumin alone at concentrations of 25 μ M and above had a marked (60–95%) inhibitory effect on HCEC cell growth, whereas

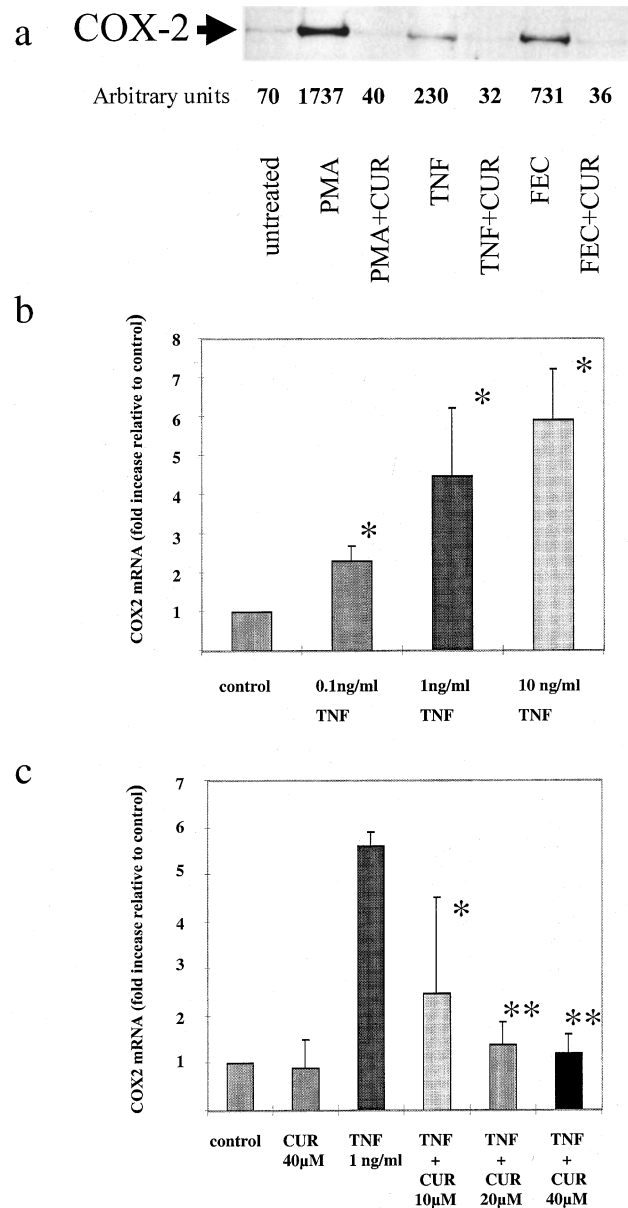


Figure 1 Effects of curcumin (CUR) on COX2 expression by PMA, TNF α or fecapentaene-12 (FEC) in HCEC cells. (a) Western blot of COX2 in control (vehicle treated) HCEC cells or cells treated as indicated with 50 ng/ml PMA, 50 ng/ml PMA + 20 μ M curcumin, 10 ng/ml TNF α , 10 ng/ml TNF α + 20 μ M curcumin, 40 μ M fecapentaene-12 + 20 μ M curcumin for 6 h. Western blot data are representative of three separate experiments. Arbitrary units represent the sum of the pixel values in the band minus the background. (b) Effect of TNF α on expression of COX2 mRNA in control (vehicle treated) HCEC cells and cells treated as indicated with TNF α (0.1–10 ng/ml) for 2 h. Levels of mRNA were determined by RT-PCR. *Significantly higher than untreated cells as determined by Student's *t*-test, $p > 0.05$. (c) Effect of curcumin on induction of COX2 mRNA by TNF α in control (vehicle treated) HCEC cells and cells treated as indicated with curcumin (40 μ M), TNF α (1 ng/ml) or TNF α + curcumin (10–40 μ M). Results are means \pm s.d. of three measurements performed in three separate experiments. **, **Significantly lower than TNF α alone as determined by Student's *t*-test, $P < 0.05$, $P < 0.001$

10 μ M had little or no inhibitory effect (data not shown).

Since the promoter region of *COX2* contains two NF- κ B binding sites, and since TNF α is a potent activator of NF- κ B in many cell types, and fecapentaene-12 has been shown to act as a cofactor for protein kinase C (Hoshina *et al.*, 1991), which is known to activate NF- κ B (Diaz-Meco *et al.*, 1993), we used electrophoretic mobility shift assays (EMSA) to examine the ability of curcumin to block NF- κ B activation by these agents. After treatment of cells with either TNF α (1 ng/ml) or fecapentaene-12 (20 μ M), there was a marked induction of nuclear protein binding to an oligonucleotide containing the 'consensus' NF- κ B binding sequence (Figure 2a, lane 2; Figure 2b, lane 14). Pretreatment of cells with curcumin (10–40 μ M) caused an inhibition of protein-DNA binding induced by either TNF α (Figure 2a, lanes 3–5) or fecapentaene-12 (Figure 2b, lane 15). To eliminate the possibility that the inhibitory effects of curcumin on NF- κ B-DNA binding could be attributed to its antioxidant properties, we tested the ability of caffeic acid phenyl ester (CAPE), an antioxidant which is structurally related to curcumin, to inhibit binding. CAPE (10–40 μ M) had no significant inhibitory effect on the induction of protein-DNA binding induced by TNF α (Figure 2a, lanes 6–8), nor did it affect the induction of *COX2* mRNA levels by either tumour promoter (Figure 3a). Similar results were obtained when N-acetyl cysteine (5 mM), a classical antioxidant, was substituted for CAPE (Figure 3b). These results suggest that curcumin inhibited activation and translocation of NF- κ B to the nucleus in a manner unrelated to its antioxidant capacity.

To assess the ability of curcumin to inhibit NF- κ B transactivation, we performed transient transfection assays with an NF- κ B-luciferase reporter construct (p6NF- κ B), containing 6 NF- κ B consensus sequences. As HCEC cells displayed poor transfection efficiency,

we used the colon carcinoma cell line SW480 for these studies. Exposure of these cells, transiently transfected with p6NF- κ B and a β -galactosidase plasmid pCMVgal, to TNF α (10 ng/ml) caused a threefold induction of p6NF- κ B luciferase activity (Figure 4, column 6). Curcumin (20 μ M) blocked this induction of luciferase activity (column 8), indicating that it inhibits the transactivating potential of NF- κ B.

To determine whether curcumin would inhibit the phosphorylation and degradation of the NF- κ B sequestering protein I κ B, we performed Western blot analyses with I κ B-specific antibody. Curcumin completely blocked the degradation of I κ B caused by TNF α (10 ng/ml) in HCEC cells (Figure 5, lanes 3–5). Instability of I κ B is thought to be controlled by phosphorylation via the NIK/IKK signalling complex (Woronicz *et al.*, 1997). To test the possibility that curcumin acts through inhibition of this complex, we measured its ability to inhibit NF- κ B-mediated alkaline phosphatase reporter gene activity (p4NF- κ B), induced either by exposure to TNF α (10 ng/ml) or by overexpression of the NIK kinase, following transfection of a NIK expression construct (pcDNA3-NIK). These experiments were carried out in HEK293 human embryonic kidney cells, to circumvent the very poor transfection efficiency in HCEC cells. Curcumin (20 μ M) inhibited TNF α -mediated induction of p4NF- κ B alkaline phosphatase activity by \sim 60% (Figure 6a) and a similar inhibition (\sim 40%) was observed following overexpression of NIK (Figure 6b), suggesting that curcumin acts via inhibiting NIK or kinases downstream of NIK, namely IKK α or β . Inhibition in this system was less pronounced than the inhibition of I κ B degradation observed in Western blot experiments with pNF- κ B6 (see above), because curcumin was added after transfection and overexpression of NIK in order to prevent it from interfering with the transfection *per se*. When we performed the same transfection experiments in SW480 colon carcinoma

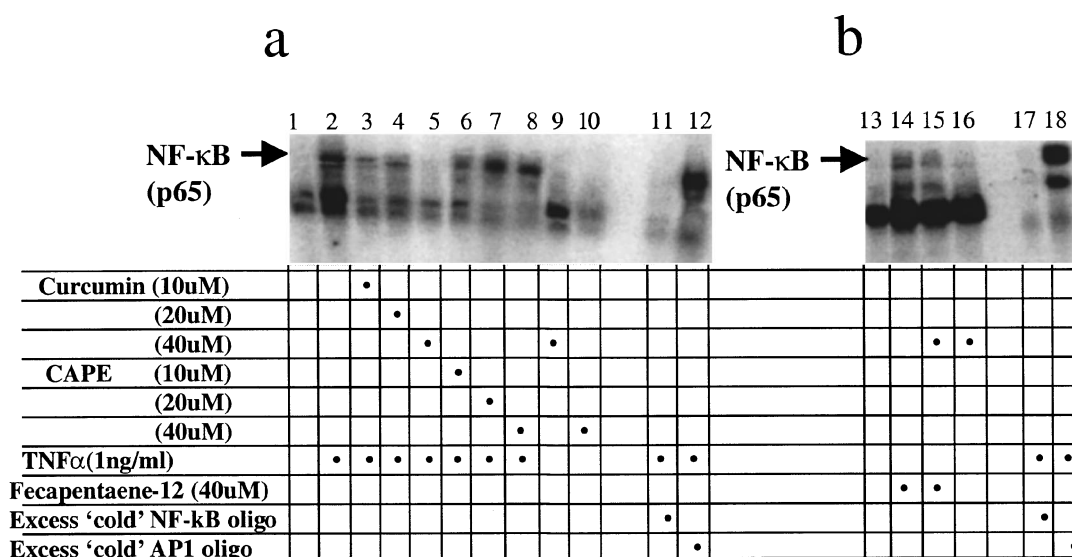


Figure 2 Effects of curcumin on NF- κ B DNA binding to 'consensus' NF- κ B oligonucleotides. Nuclear proteins were extracted from HCEC cells and incubated for 40 min on ice with end-labelled oligonucleotides prior to electrophoresis. (a) EMSAs with NF- κ B 'consensus' oligonucleotide in HCEC cells treated as indicated with vehicle (control), 1 ng/ml TNF α , TNF α + curcumin (10 μ M–40 μ M), TNF α + CAPE (10–40 μ M), 40 μ M curcumin alone or 40 μ M CAPE alone; (b) vehicle (control), 20 μ M fecapentaene-12, fecapentaene-12 + 20 μ M curcumin, or 20 μ M curcumin alone

cells, which had been pretreated with curcumin for 1 h prior to exposure to TNF α , the induction of p6NF- κ B reporter gene activity was inhibited by greater than 90% (Figure 4).

In a similar transfection experiment using a construct, pFLAG-IKK α , to overexpress IKK α , or pFLAG-IKK β to overexpress IKK β , curcumin was again able to inhibit the reporter gene activity of the NF- κ B co-transfected reporter constructs by ~45 and ~60% respectively (Figure 6c). Salicylate (10 mM) also inhibited the IKK α and IKK β overexpression mediated

activation of the NF- κ B reporter by ~45 and ~70%, respectively (Figure 6d).

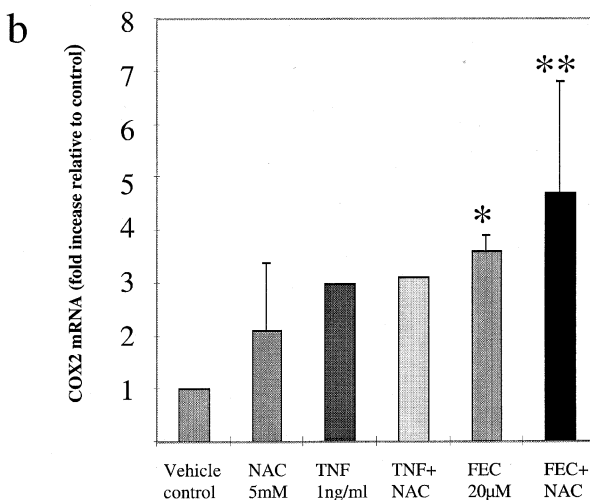
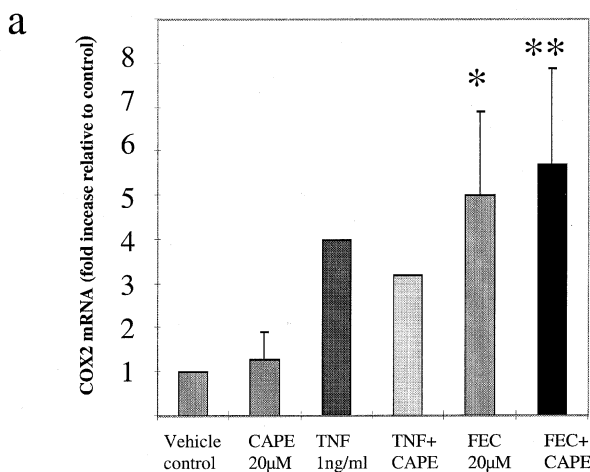


Figure 3 Effect of CAPE or N-acetyl cysteine (NAC) on COX2 mRNA levels following exposure of HCEC cells to TNF α or fecapentaene-12 (FEC). (a) Lack of inhibition of COX-2 mRNA induction by CAPE in HCEC cells treated as indicated with vehicle (control), CAPE 20 μ M, TNF α (1 ng/ml), TNF α +CAPE (20 μ M), fecapentaene-12 (20 μ M), fecapentaene-12 (20 μ M)+CAPE (20 μ M); (b) Lack of inhibition of COX-2 mRNA induction by N-acetylcysteine (NAC) in HCEC cells treated as indicated with vehicle (control), NAC (5 mM), TNF α (1 ng/ml), TNF α (1 ng/ml)+NAC (5 mM), fecapentaene-12 (20 μ M), fecapentaene-12 (20 μ M)+NAC (5 mM). Results are means (\pm range) of measurements performed in two different experiments. For TNF α exposures results are from a single experiment. Similar results were obtained with TNF α at 10 ng/ml (data not shown). *Significantly higher than control ($P < 0.05$), **not significantly different from fecapentaene alone, as analysed by Student's *t*-test

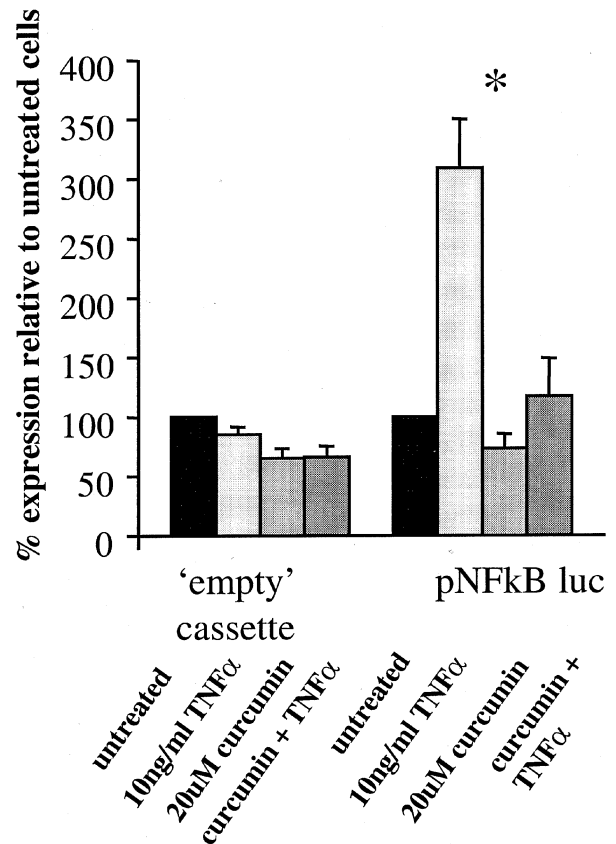


Figure 4 Inhibition of TNF α -induced NF- κ B dependent transactivation by curcumin. SW480 cells were transfected with the plasmid tk-36-LUC ('empty' cassette), columns 1–4, or p6NF- κ B-tk-LUC, columns 5–8 and treated as indicated with vehicle (control), TNF α (10 ng/ml), curcumin alone (20 μ M) or TNF α (10 ng/ml)+curcumin (20 μ M) prior to measuring luciferase reporter gene activity. Values represent the means \pm s.d. of three separate transfections carried out in duplicate. *Significantly higher than untreated sample as analysed by Student's *t*-test, $P < 0.01$

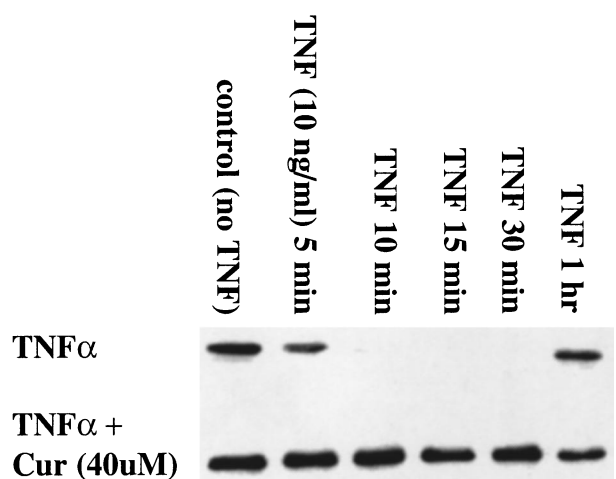


Figure 5 Effect of curcumin on TNF α -induced I κ B degradation. HCEC cells were treated as indicated with 10 ng/ml TNF α for various times (5 min–1 h) in the absence or presence of 20 μ M curcumin which was added to the media 1 h prior to TNF α . Cytosolic proteins were subjected to analysis by Western blotting using an I κ B α specific antibody (Santa Cruz)

Discussion

Our results demonstrate that the dietary constituent curcumin inhibits activation of NF- κ B which in turn inhibits expression of the *COX2* gene induced by the tumour promoters PMA, TNF α or fecapentaene-12 in

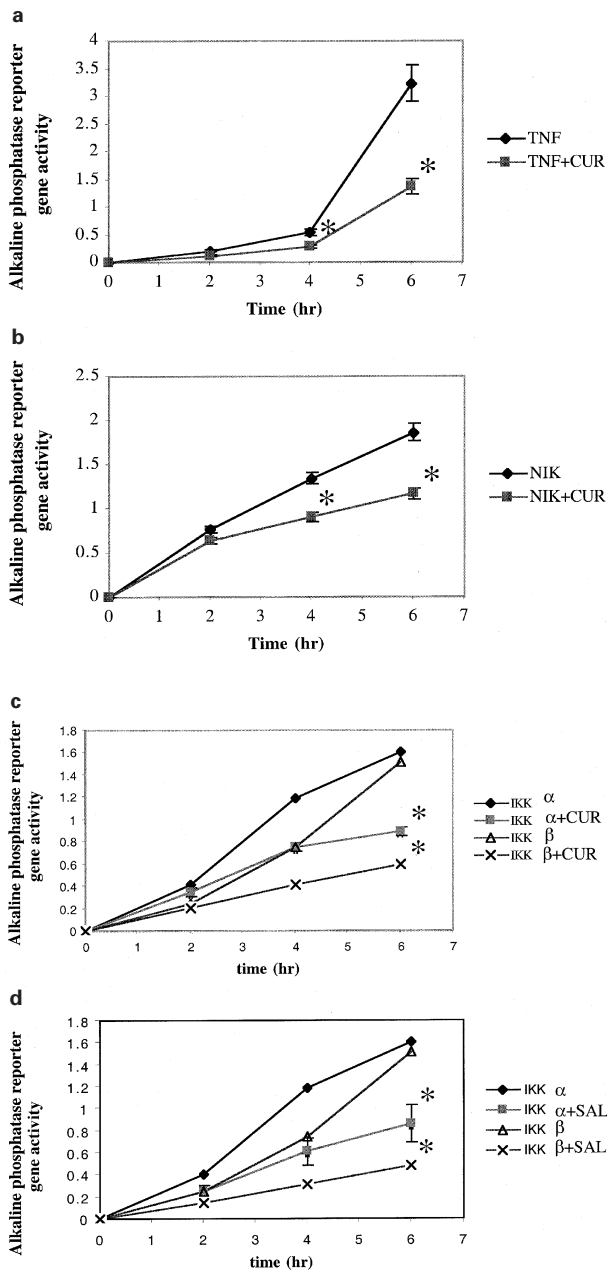


Figure 6 Effect of curcumin or salicylate on the activity of components of the NIK/IKK complex. HEK293 cells were transfected with NIK, IKK α or IKK β expression constructs in combination with an NF- κ B reporter construct or with the reporter construct alone. Following the dual transfections (b,c,d) cells were exposed to curcumin (20 μ M), salicylate (10 mM) or vehicle control prior to measuring alkaline phosphatase reporter gene activity at 2, 4 and 6 h after the addition of inhibitor to the medium. Cells transfected with the reporter construct alone (a) were exposed to TNF α (10 ng/ml), after a 1 h pretreatment with curcumin (20 μ M) and alkaline phosphatase reporter gene activity was measured at 2, 4 and 6 h after the addition of TNF α . Results represent the mean \pm s.e.m. and are representative of at least three separate experiments. *Significantly different from reporter gene activity measured in the absence of curcumin or salicylate as determined by balanced ANOVA analysis, $P < 0.05$

human colon epithelial cells. The data suggest that curcumin blocks tumour promoter-mediated NF- κ B transactivation by inhibiting the NIK/IKK signalling complex, probably at the level of IKK α/β . These data are consistent with the recent observations of Morteau *et al.* (1998) who have shown that NF- κ B is critical for the induction of COX-2 gene expression by TNF α in human colon tumour cells. Overexpression of COX2 in colon epithelial cells, which occurs during colon carcinogenesis, causes resistance to apoptosis (Tsuji and Dubois, 1995), suggesting that treatment with curcumin might reinstate susceptibility to apoptosis. This interpretation is consistent with the observation that curcumin increases the percentage of apoptotic cells in colon tumours of rats exposed to azoxymethane, while decreasing tumour incidence by 50% (Samaha *et al.*, 1997). NF- κ B activation prevents TNF α -induced cell death by blocking apoptosis (Beg and Baltimore, 1996). It is therefore possible that COX2 is one of the downstream mediators of this effect as previously suggested (Zeng-gang *et al.*, 1996).

The magnitude of inhibition of p4NF- κ B reporter gene activity by curcumin was similar whether the reporter gene induction was driven by overexpression of NIK, IKK α or IKK β or by addition of TNF α , suggesting that curcumin has little or no inhibitory effect at the level of the TNF receptor. It is unlikely that the inhibitory effects of curcumin on the NIK/IKK complex are due to non-specific antioxidant activity, since neither CAPE nor N-acetyl cysteine had any effect on NF- κ B (p65)-DNA binding induced by the tumour promoters. Higher concentrations of CAPE than those used in this study have been shown to inhibit TNF α -mediated NF- κ B-DNA binding in U937 cells, but probably through blocking DNA binding directly, since there was no inhibition of I κ B degradation (Natarajan *et al.*, 1996).

It is unlikely that fecapentaene-12 activates the NIK/IKK pathway at the level of the TNF receptor, but we have shown previously that it induces oxidative stress in cells (Plummer and Faux, 1994). As reactive oxygen intermediates are a common denominator in NF- κ B activating signals, they may be involved with the effects of fecapentaene-12 on the NIK/IKK pathway. However, as already mentioned, fecapentaene-12 can act as a cofactor for protein kinase C (Hoshina *et al.*, 1991), which is also known to activate NF- κ B (Diaz-Meco *et al.*, 1993). A similar mechanism may be operated by PMA (Ghosh and Baltimore, 1990). Both TNF and fecapentaene-12 activate AP1 (Zeng-gang *et al.*, 1996; Holloway *et al.*, 1998), which is important in the induction of *COX2* transcription by v-Src (Xie and Herschman, 1995) and PMA (Subbaramaiah *et al.*, 1998). Since curcumin inhibits AP1 dependent transactivation (Huang *et al.*, 1991), some of the inhibitory effects of curcumin on *COX2* gene induction by the tumour promoters may also be mediated by inhibition of AP-1 (Kelley *et al.*, 1996).

The ability to inhibit the NIK/IKK signalling complex may be common to the action of other anti-inflammatory chemopreventive agents. Aspirin and salicylate, shown previously to inhibit I κ B phosphorylation and degradation (Kopp and Ghosh, 1994), have recently been shown to inhibit the phosphorylation of I κ B by specifically reducing ATP binding to IKK β (Yin *et al.*, 1998). Salicylate was also shown to

inhibit *COX2* induction by LPS in macrophages (Tordman *et al.*, 1995) and endothelial cells (Du *et al.*, 1998) and our unpublished immunofluorescence data is consistent with this observation. Yin *et al.* (1998) have shown that salicylate inhibits IKK β selectively in *in vitro* kinase assays. In contrast our studies show that salicylate can inhibit NF- κ B activation by IKK α overexpression in addition to its inhibition of IKK β -mediated NF- κ B activation, albeit to a lesser extent. The reason for this apparent discrepancy may reflect the fact that in our overexpression system the ability of IKK α to activate NF- κ B is in some way dependent on IKK β and that the effects of both inhibitors are mediated through inhibition of this latter kinase.

Whether or not to use aspirin as a cancer chemopreventive agent in large numbers of individuals at risk of developing colon cancer is currently hotly debated (Vainio, 1997; Trujillo *et al.*, 1994), in view of the possible toxicity associated with chronic aspirin administration. If curcumin can be shown to have chemopreventive activity against colon carcinogenesis in human clinical trials, comparable to that already demonstrated unequivocally in animal models of this disease, its long history of dietary consumption without adverse health effects might make it an important alternative to aspirin for chemoprevention of this disease. It is difficult to assess the potential biological relevance of the concentrations of curcumin used in this study since the bioavailability of curcumin in the colonic epithelium is not known, and since access to the colon does not necessarily require systemic absorption, a comparison with plasma concentrations may be misleading. Studies to assess the bioavailability of curcumin in humans are ongoing.

In this study we have shown that curcumin inhibits the induction of *COX2* in human colonic cells by tumour promoters that occur in the human colon. Since overexpression of this enzyme is probably important in the pathogenesis of human colon cancer, our data also suggest that measurement of the effects of curcumin on *COX2* gene expression may be a useful surrogate biomarker for the assessment of its biological activity in chemoprevention trials. Finally, our finding that curcumin acts in part through inhibition of the NIK/IKK signalling cascade provides a focus for the rational development of novel chemopreventive agents.

Materials and methods

Cell culture

Human colon epithelial cells (HCEC) and SW480 colon carcinoma cells were kindly provided by Dr Andrea Pfeifer (Nestec Ltd. Research Centre, Lausanne, Switzerland) and Professor Christos Paraskeva (Bristol University), respectively. Cells were grown in Dulbecco's minimal essential medium (DMEM) (Gibco-BRL Ltd.), supplemented with 10% foetal calf serum (FCS). For HCEC cells tissue culture vessels were pre-coated with medium containing 10 μ g/ml Vitrogen 100 (Collagen Corp.), 2.5 μ g/ml human fibronectin (Sigma) and 50 μ g/ml BSA (Gibco-BRL) prior to plating. Fecapentaene-12 (SR1 International, Menlo Park, CA, USA), TNF α (Sigma), curcumin (Sigma) and sodium salicylate (Sigma) were diluted to concentrations of 10–40 μ M, 0.1–10 ng/ml, 10–40 μ M and 1 mM, respectively, in DMEM. In

incubations containing curcumin or salicylate, cells were pretreated for 1 h with these agents prior to the addition of the tumour promoters. Curcumin stock solutions were made up in dimethylsulphoxide (DMSO) immediately before each experiment in light-impervious tubes. The handling and storage of fecapentaene-12 was carried out according to a previously described procedure (Plummer and Faux, 1994). The viability of cells was determined by the MTT assay (Scudiero *et al.*, 1988). Protein assays were performed using the Bradford reagent (Biorad) or the Lowry method (Sigma).

Western blot analysis

Cells were washed with buffer (20 mM Tris base, 150 mM NaCl, 5 mM glucose, 2 μ g/ml leupeptin and 20 μ g/ml aprotinin, pH 7.4), lysed in homogenisation buffer (20 mM Tris HCl, 2 mM EDTA, 2 mM EGTA, 6 mM β -mercaptoethanol, 2 μ g/ml leupeptin and 2 μ g/ml aprotinin, pH 7.5), sonicated for 20–30 s and centrifuged at 12 000 *g* for 35 min at 4°C. After electrophoresis, proteins were electroblotted to nitrocellulose, the membrane was blocked with 10% Marvel and 0.1% Tween 20 for 2 h at room temperature, incubated with primary antibody in blocking buffer (10 mM Tris, 0.14 M NaCl, 5% milk powder (Marvel) pH 7.6) overnight at room temperature and then horseradish peroxidase secondary antibody diluted 1:1500 (v/v) in blocking buffer for 1.5 h at room temperature, before visualisation by the ECL method (Amersham). Rabbit polyclonal antibodies raised against *COX2* and I κ B α (Santa Cruz Biotechnology Inc.) were used at 1:1000 dilution (v/v) in blocking buffer. Visualisation and quantitation of bands was carried out by a scanning densitometer (Molecular Dynamics) using ImageQuant software.

RT-PCR

Semi-quantitative RT-PCR analysis of *COX2* mRNA levels was performed according to the method of Hla and Maciag (1991). Briefly, 1 μ g of RNA, extracted from cells using TRIzol reagent (Gibco-BRL), according to the manufacturer's instructions, was reverse transcribed in 10 μ l RT buffer containing MMLV reverse transcriptase (10 u/ μ l), RNasin (Gibco-BRL) (1 u/ μ l), dATP, dGTP, dTTP and dCTP (1 mM), random hexamers (Boehringer Mannheim) (15 pmoles/ μ l), MgCl₂ (5 mM) and dithiothreitol (DTT) (1 mM). The reaction product was diluted 1 in 10 with distilled water and 10 μ l of this subjected to PCR by adding 40 μ l PCR buffer containing *COX2* or GAPDH primers (1.25 pmoles/ μ l), MgCl₂ (2.5 mM), dATP, dGTP, dCTP, dTTP (1 mM) and Taq DNA polymerase (Gibco/BRL) (0.2 u/ μ l) and heating for 4 min at 94°C followed by 25 (*GAPDH* primers) or 25 (*COX2* primers) cycles of 94°C 1 min, 58°C 1 min and 72°C 1 min. PCR reaction products were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed prior to densitometric measurements using a Molecular Dynamics computing densitometer. The amount of amplified product was confirmed by this method to be linear with respect to the input RNA for both *COX2* and *GAPDH* primers. The densities of the *COX2* bands were normalised with respect to the *GAPDH* bands in parallel PCR reactions.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were made according to the method of Staal *et al.* (1990) from 5×10^6 HCEC cells. EMSA were performed using the NF- κ B 'consensus' oligonucleotide- 5'-AGTT-GAGGGGACTTTCCAGGC-3'. Nuclear protein extract (4 μ g) was incubated with 0.25 pmoles ³²P-end-labelled oligonucleotide in binding buffer containing 20 mM Hepes (pH 7.5), 4% ficol, 0.5 μ g/ml poly DIDC, 0.1 mM MgCl₂ and

0.1 mM DTT, on ice for 40 min. The DNA: protein complex formed was separated from free oligonucleotide on a 4% non-denaturing polyacrylamide gel. Following electrophoresis, the gel was dried and visualization and quantitation of radioactive bands performed by a PhosphorImager (Molecular Dynamics) using image-quant™ software. Specificity of binding was checked by incubating in the presence of an excess of 'cold' NF- κ B oligo or an unrelated oligo containing an API 'consensus' DNA binding sequence – 5'-GCTTGATGAGTCAGCCGGAA-3' (Promega).

DNA transfections

SW480 cells (1.6×10^7) were transfected in serum free DMEM with 1.25 pmoles of the plasmid p6NF- κ B-tk-LUC (p6NF- κ B) (kindly provided by Dr Patrick Baeuerle, University of Freiburg) and 0.1 pmoles pCMVB (Promega) by electroporation. The 'empty' cassette (tk-36-LUC) was used as a negative control. The plasmid pCMVB was cotransfected with the other constructs to enable normalisation of luciferase activity to β -galactosidase activity, thus controlling for differences in transfection efficiency. Following transfection, cells were resuspended in DMEM containing 10% FCS and allowed to recover for 5 h. Cells were then switched to DMEM containing 1% FCS for 24 h prior to exposure to TNF α or fecapentaene-12 at final concentrations of 10 ng/ml or 20 μ M, respectively, for 2 h in the absence or presence of 20 μ M curcumin. Curcumin was added in DMEM with 2% FCS 1 h prior to the TNF/fecapentaene-12 exposures. Luciferase and β -galactosidase enzyme activities were measured using Promega assay kits with a Wallach MicroBeta 1450 plate reader or Labsystems iEMS reader, respectively. Luciferase activity was expressed in relative units after normalisation to β -galactosidase

HEK 293 cells were transfected with NIK (pcDNA3-NIK), IKK α (pFlag-IKK α) or IKK β (pFlag-IKK β) expression constructs (I κ B kinase constructs were kindly provided by David Goeddel) together with an NF- κ B alkaline phosphatase reporter construct (p(NF- κ B)4-tk-sPAP) or with p(NF- κ B)4-tk-sPAP alone using Fugene (Boehringer) according to the manufacturer's instructions. After 18 h cells were incubated in the absence or presence of curcumin (20 μ M). pNF- κ B4-tk-sPAP transfectants were incubated with TNF α (10 ng/ml) 1 h prior to the addition of the curcumin-

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containing medium. All transfections included either the β -lactamase expression construct pRSV-lactamase or the β -galactosidase expression construct pRSV β -Gal to assess transfection efficiency. Measurements of alkaline phosphatase reporter gene activity were made at various times after addition of curcumin to the medium.

Statistics

RT-PCR data were analysed using the Student's *t*-test. Transfection data were analysed by Student's *t*-test or balanced ANOVA combined with Fisher's test.

Abbreviations

NF- κ B, nuclear factor kappa B; COX-2, cyclo-oxygenase-2; TNF α , tumour necrosis factor alpha; FEC, fecapentaene-12; PMA, phorbol 12-myristate 13-acetate; CUR, curcumin; SAL, salicylate; CAPE, caffeic acid phenyl ester; NAC, N-acetyl cysteine; NIK, NF- κ B inducing kinase; IKK, I kappa B kinase; EMSA, electrophoretic mobility gel shift assay; ANOVA, analysis of variance.

Note added in proof

A preliminary report of this work is published as an abstract: Plummer SM, Holloway KA, Munks RJJ, Manson MM, Kaptein A, Farrow S and Hubbard A. (1999). The chemopreventive agent curcumin suppresses cyclooxygenase-2 expression in colon cells by inhibiting the NIK/I κ B kinase activation of NF- κ B. *Br. J. Cancer* (in press).

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