Curcumin induces c-jun N-terminal kinase-dependent apoptosis in HCT116 human colon cancer cells

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Curcumin, the major pigment of the dietary spice turmeric has the potential for chemoprevention by promotion of apoptosis. Mitogen-activated protein kinase (MAPK) and NF-kappa B (NFkB) signalling cascades are thought to regulate apoptosis and cell survival. While curcumin inhibits NFκB, its effects upon the MAPK pathways are unclear. This study investigates curcumin effects upon MAPK signalling and apoptosis in HCT116 cells. Here we report that curcumin time- and dose-dependent induction of apoptosis were accompanied by sustained phosphorylation and activation of c-jun N-terminal kinase (JNK) and p38 MAPK as well as inhibition of constitutive NFκB transcriptional activity. Curcumin treatment also induced JNK-dependent sustained phosphorylation of c-jun and stimulation of AP-1 transcriptional activity. Curcumin-mediated c-jun phosphorylation and apoptosis were reduced by treatment with the JNK-specific inhibitor SP600125. Conversely, the p38-specific inhibitor SB203580 had no effect upon curcumin-induced apoptosis. Curcumin treatment had no effect on the activity of extracellular signal-regulated protein kinase (ERK). Taken together, our data show for the first time that JNK, but not p38 or ERK signalling, plays an important role in curcumin-mediated apoptosis in human colon cancer cells that may underlie its chemopreventive effects.

Introduction

Curcumin (diferuloylmethane) is a naturally occurring polyphenolic pigment, isolated from the rhizomes of the plant Curcuma longa (Linn), and is commonly used as a colouring and flavouring agent in food products. Curcumin has antioxidant (1) and anti-inflammatory effects (2), promotes apoptosis in neoplastic cells in vitro (3,4), in intestinal epithelium in vivo (5,6) and is a potent inhibitor of carcinogen-induced tumorigenesis in rodent intestine (5,7). Curcumin consumption at up to 100 mg/day in certain countries (8) testifies to its pharmacological safety and the agent is currently undergoing phase I clinical trials for cancer prevention (9). Curcumin shows some cell-type specificity of effects. For example, it induces p53-dependent apoptosis in MCF-7 (10) and basal carcinoma cells (11) whereas in melanoma cells this process is independent of p53 (12). In colonic epithelium, curcumin has been shown to inhibit nuclear factor kappaB (NFκB) activity (13), cyclooxygenase-2 expression (14) and reduce β-catenin transactivation (15), which may all play a role in curcumin-mediated apoptosis.

Certain polyphenolic compounds may induce apoptosis through modulation of the mitogen-activated protein kinases (MAPK) pathways (16-18). c-jun N-terminal kinase (JNK) is a member of the MAPK family (19) that is activated by inflammatory cytokines and a variety of chemical stresses. Activated JNK phosphorylates and activates c-jun and other transcription factors such as activating transcription factor 2 (ATF-2) and Elk-1 but its role in apoptosis remains controversial. Activation of JNK may have pro-apoptotic (20-23) or anti-apoptotic effects (24,25) and may also promote cell proliferation (26). Similarly, c-jun, the major downstream effector of JNK, has also been shown to display both pro- and anti-apoptotic properties (27,28). Recent work has shown that inhibition of the transcription factor NFκB results in sustained activation of JNK after TNF treatment. Sustained JNK activity was found to be pro-apoptotic, whereas rapid, transient activation could be anti-apoptotic (25,29,30). However, conflicting evidence suggests that sustained JNK activity in the absence of NFκB can inhibit apoptosis (31).

Certain dietary chemicals with chemopreventive properties such as capsaicin (32) and isothiocyanates (33,34) have been shown to induce apoptosis through a JNK-dependent mechanism. Curcumin inhibits NFκB activation and has been shown previously to reduce JNK (35) and AP-1 transcriptional activity (36) induced by cytokine stimulation. However, data relating to effects of curcumin alone on JNK activation are lacking. We hypothesized that curcumin-induced apoptosis may involve sustained activation of JNK in addition to inhibition of constitutive NFκB activity and investigated these themes in HCT116 human colon cancer cells. This cell line provides a suitable model of early APC/K-Ras-mediated neoplastic transformation relevant to colorectal cancer. For example, HCT116 cells have intact APC (37) but contain one wild-type allele and one mutant allele for an activating mutation of β-catenin (CTNNB1WT/Δ45) (38). HCT116 cells also contain mutationally activated K-ras (39) with intact JNK, Raf-extracellular signal-regulated kinase (ERK) (40) and p38 responses (41). In addition, HCT116 cells retain wild-type inducible p53 and NFκB responsiveness (42). Here, we show that curcumin promotes activation of JNK and c-jun, and stimulates AP-1 transcriptional activity. Blockade of these effects by a JNK-specific inhibitor impedes curcumin-induced apoptosis. These studies suggest a novel role for JNK effector pathways in curcumin-mediated apoptosis.

Materials and methods

Materials
Curcumin was 80% pure (98% curcuminoid content) and was obtained from Sigma-Aldrich, Poole, Dorset, UK. SP600125 and SB203580 were purchased...
from Biomol Research Laboratories, Plymouth Meeting, PA. All antibodies were obtained from New England Biolabs, Hitchin, Hertfordshire, UK. All other reagents were widely available commercially.

**Cell culture**

Human colon cancer cell line HCT116 was obtained from the European Collection of Cell Cultures (ECACC), Salisbury, UK and grown as monolayers at 37°C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle's medium containing 10% (v/v) heat inactivated fetal calf serum, glucose (2 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml). Curcumin and the two inhibitors SP600125 and SB203580 were dissolved in dimethyl sulfoxide (DMSO). Controls were treated with 0.1% DMSO alone. All treatments were carried out on cells at 60-80% confluence.

**Western blotting**

Cells at 60-80% confluence were washed with PBS and lysed directly into SDS-PAGE loading buffer. Soluble protein (30 μg) was resolved by SDS-PAGE and transferred to nitrocellulose membrane. For all experiments, equal protein loading was confirmed by staining the nitrocellulose with Ponceau S. Membranes were probed with the appropriate primary antibodies. Anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 (Thr180/Tyr182) and anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibodies were obtained from New England Biolabs. All antibodies were non-cross-reactive with the corresponding phosphorylated residues of other MAP kinase family members. All primary antibodies were used at 1:1000 in 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween. Reactions were visualized with a suitable secondary antibody conjugated to horseradish peroxidase (Dako) using an enhanced chemiluminescence system (Santa Cruz, CA).

**Assay for p38 activity**

p38 activity was assessed using a non-radioactive p38 MAP kinase assay kit (New England Biolabs) according to the manufacturer’s instructions. Briefly, activated p38 was immunoprecipitated by incubating cell lysates overnight with an immobilized phospho-p38 antibody. An *in vitro* kinase assay was then performed using an ATF-2 fusion protein as substrate. Levels of phosphorylated ATF-2 were then detected by western blotting using a phospho-ATF-2 (Thr71) antibody.

**Cell viability assay**

Cells were seeded at 1 × 10⁴ cells/well in 96-well plates then treated with curcumin with or without MAP kinase inhibitors for 24 h. At the time points shown 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (20 μl) was added and incubated for 1 h. Cells were then incubated overnight with 20% SDS and the formation of coloured formazan dye was assessed colorimetrically at 550 nm. Results are expressed as percentage loss of cell viability compared with control.

**In situ end-labelling (ISEL) assay**

ISEL assays were carried out essentially as described previously (5). In brief, cells were grown in Labtek II chamber slides. After fixing in ice-cold methanol, slides were stored at -20°C until further analysis. Cells were re-hydrated and incubated with 1 μg/ml protease K for 30 min at 37°C, followed by incubation with 10 μM each of dCTP, dGTP, dTTP and biotinylated dATP and biotinylated dTTP and incubated at 37°C for 30 min. To visualize apoptotic cells, the slides were incubated with streptavidin–biotin complex (Dako) and colour development was performed with 3,3'-diaminobenzidine. Apoptotic cells were identified by their brown colouration. The percentage of apoptotic cells was calculated from a total of 300 cells in randomly chosen fields.

**Transient transfections and luciferase assay**

Transfection was performed in 24-well plates using Lipofectin reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were grown in 24-well plates and transfected with the appropriate vector (250 μg) the following day. After 5 h the transfection mix was removed and replaced with complete medium. Treatment of cells with curcumin or vehicle was then carried out 24 h after transfection. Luciferase activity was determined using the luciferase assay system with reporter lysis buffer (Promega). Results were expressed as relative luciferase activity and were corrected for differences in transfection efficiency by co-transfection with a pCMVβGal construct followed by β-galactosidase assay (Promega).

**Statistical analysis**

Data are presented as mean ± SD. Comparisons between groups were made with the paired Student’s *t*-test. Values of *P* < 0.05 were considered statistically significant.

**Results**

**Curcumin induces apoptosis in HCT116 cells**

To assess the effect of curcumin on cell viability we exposed HCT116 cells to a dose range of curcumin over time intervals up to 24 h and performed MTT assays. Curcumin treatment resulted in a time- and dose-dependent loss of cell viability as shown in Figure 1A. In order to determine whether this cell death was apoptotic, whole cell lysates from curcumin-treated cells were assessed by western blotting using an antibody that recognizes both the intact (116 kDa) and cleaved (89 kDa) forms of poly (ADP-ribose) polymerase (PARP). Cleavage of PARP is a hallmark of apoptotic cell death. As shown in Figure 1B, exposure of HCT116 cells to curcumin resulted in a time-dependent cleavage of PARP. Furthermore, ISEL analysis revealed a time-dependent induction of apoptosis by curcumin (Figure 1C).

**Curcumin inhibits constitutive NFκB transcriptional activity and stimulates the JNK and p38 pathways in HCT116 cells**

Curcumin has been shown previously to inhibit constitutive NFκB activity in a number of cell types. In order to determine the effect of curcumin on NFκB activity in HCT116 cells we transiently transfected cells with the 3EnhConAluc reporter vector. This construct carries the luciferase gene under the control of three copies of the kappa B consensus of the immunoglobulin κ-chain promoter located upstream of the conalbumin transcription start site (43). Curcumin (35 μM) treatment resulted in a significant reduction in NFκB-dependent luciferase activity (Figure 2A). To determine the effect of curcumin on the activation of MAP kinase signalling pathways, we assessed whole cell lysates from curcumin-treated cells by western blotting analysis using antibodies that specifically recognize the phosphorylated and activated forms of JNK, p38 and ERK. As shown in Figure 2B, exposure of HCT116 cells to curcumin resulted in sustained phosphorylation and activation of both JNK and p38. Activation of both kinases was observed 4 h after the start of curcumin treatment, and was sustained for at least 24 h. ERK was revealed to be constitutively activated in HCT116 cells and curcumin treatment had no effect on the levels of phosphorylated ERK. Western bloting with antibodies that recognize JNK and p38 regardless of their phosphorylation state revealed that the overall levels of these proteins were not affected by curcumin. The activation of JNK by curcumin was confirmed by western blotting of whole cell lysates using an antibody specific to the phosphorylated form of the JNK substrate c-jun. Phosphorylation of c-jun occurred over the same sustained period as JNK activation (Figure 2B). Similarly, p38 activation was further assessed by an immunocomplex kinase assay using an ATF-2 fusion protein as substrate followed by western blotting using an antibody specific to phosphorylated ATF-2. Curcumin treatment resulted in sustained p38 kinase activity over the same period as p38 phosphorylation (Figure 2B).

**Curcumin induces AP-1 transcriptional activity**

The most well characterized substrate for phosphorylation by JNK is c-jun. Cellular stress stimuli, which result in JNK activation ultimately result in activation of c-jun by phosphorylation at Ser-63 and Ser-73 within the c-jun transactivation domain. Activated c-jun homodimerizes or forms a heterodimer with c-fos and binds to AP-1 response elements in the promoters of target genes. Having established that curcumin...
activates JNK and leads to subsequent phosphorylation of c-jun in HCT116 cells, we sought to determine the effect of curcumin on AP-1 transcriptional activity by transfecting cells with an AP-1 luciferase reporter construct followed by treatment with curcumin (35 μM) for 24 h. Figure 3 shows that curcumin treatment resulted in a 2.7-fold increase in AP-1 transcriptional activity.

**Inhibition of JNK confers resistance to curcumin-induced apoptosis**

To investigate whether the JNK and p38 pathways are required for curcumin-induced apoptosis, we co-treated HCT116 cells with curcumin and either the specific JNK inhibitor SP600125 (44), the specific p38 inhibitor SB203580 (45), or a combination of both. The specificity of these inhibitors was assessed by western blotting of whole cell lysates for phosphorylated c-jun as a measure of JNK activity, and by immunocomplex kinase assay as a measure of p38 activity. Figure 4A shows that treatment with SP600125 resulted in an inhibition of curcumin-induced JNK activation but did not interfere with p38 activity. Conversely, SB203580 treatment blocked p38 activation after exposure to curcumin, whilst having no effect on the activity of JNK. The effects of inhibition of JNK and p38 on curcumin-induced cell death were assessed by MTT assay and PARP cleavage assay. As shown in Figure 4B, inhibition of JNK resulted in a significant increase in cell viability after curcumin treatment compared with exposure to curcumin alone, whereas inhibition of p38 had no effect. Similarly, JNK inhibition resulted in a significant decrease in PARP cleavage (Figure 4C and D). Taken together, these results show that activation of JNK, but not p38, is required for apoptotic cell death induced by curcumin.

**Discussion**

Induction of apoptosis in pre-malignant cells is a critical feature of chemopreventive agents (6). Curcumin, which has been shown to be potently anti-tumorigenic, has well-documented pro-apoptotic properties in a number of different cell types, including colon cells (46). Signal transduction pathways involved in growth and apoptosis are potential targets for chemopreventive agents. Previous studies have demonstrated that curcumin inhibition of NFκB activity is accompanied by apoptosis in various cell types (47,48). Other compounds with chemopreventive properties such as selenite (49), tea polyphenols (50) and isothiocyanates (34), have been shown to exert their chemopreventive effects through modulation of MAPK
signalling pathways. Furthermore, recent evidence indicates that sustained activation of JNK may be an important mediator of apoptosis in a number of cell types, and that this process is dependent on inhibition of NFκB (29,30). These findings led us to hypothesize that JNK activation may play a role in curcumin-induced apoptosis of colon cells. Here we report for the first time that curcumin treatment of HCT116 cells results in sustained activation of JNK and induction of JNK-dependent apoptosis.

Curcumin treatment of HCT116 cells resulted in a time- and dose-dependent induction of apoptosis. For subsequent experiments we chose to use a curcumin concentration of 35 μM as higher concentrations did not result in a significant increase in cell death. Bioavailability of curcumin is generally poor after oral administration (51). Phase I clinical trials have shown that oral doses of curcumin at 8000 mg/day produced peak serum curcumin levels of only 1.77 ± 1.87 μM (52). However, levels in colonic or intestinal mucosa may be substantively higher. In an experimental study, dietary administration of 2% curcumin was associated with levels in colonic mucosa of 1.8 ± 0.8 μmol curcumin/g as opposed to only nanomolar concentrations in plasma or tissue of distant organs (53). These data support the view that higher curcumin concentrations achieved in intestine or colon after oral administration may be commensurate with greater biological activity and chemopreventive effectiveness in those tissues than in other organs (51). The in vitro curcumin concentration used in this study could be proportional to levels achieved in the intestine or colon, after dietary administration.

Curcumin-induced apoptosis was accompanied by inhibition of NFκB transcriptional activity and stimulation of JNK and p38 pathways in HCT116 cells. (A) Cells were transiently transfected with the NFκB-responsive luciferase reporter construct 3EnhConALac then treated with curcumin (35 μM) or vehicle for the times shown. Luciferase activity of cell lysates was measured and normalized to β-galactosidase activity obtained by co-transfection with a pCMVβGal internal control plasmid. Results represent the mean ± SD of three independent experiments. *P < 0.001, significantly different from control (Student’s t-test).

(B) Curcumin activates JNK and p38 signalling pathways. Cells were treated with curcumin (35 μM) for the times shown. JNK activity was determined by western blotting and by assessment of phosphorylated c-jun levels in whole cell lysates. p38 activity was assessed by western blotting and immunocomplex kinase assay as described in the Materials and methods, using an ATF-2 fusion protein as substrate. ERK activity was assessed by western blotting.

Fig. 2. Curcumin inhibits constitutive NFκB transcriptional activity and stimulates JNK and p38 pathways in HCT116 cells. (A) Cells were transiently transfected with the NFκB-responsive luciferase reporter construct 3EnhConALac then treated with curcumin (35 μM) or vehicle for the times shown. Luciferase activity of cell lysates was measured and normalized to β-galactosidase activity obtained by co-transfection with a pCMVβGal internal control plasmid. Results represent the mean ± SD of three independent experiments. *P < 0.01, significantly different from control (Student’s t-test). (B) Curcumin activates JNK and p38 signalling pathways. Cells were treated with curcumin (35 μM) for the times shown. JNK activity was determined by western blotting and by assessment of phosphorylated c-jun levels in whole cell lysates. p38 activity was assessed by western blotting and immunocomplex kinase assay as described in the Materials and methods, using an ATF-2 fusion protein as substrate. ERK activity was assessed by western blotting.

Fig. 3. Curcumin induces AP-1 transcriptional activity. Cells were transiently transfected with the AP-1-responsive luciferase reporter construct pAP-1-luc then treated with curcumin for 24 h. Luciferase activity of cell lysates was measured and normalized to β-galactosidase activity obtained by co-transfection with a pCMVβGal internal control plasmid. Results represent the mean ± SD of three independent experiments. *P < 0.001, significantly different from control (Student’s t-test).
...of its activation is a critical factor in determining cell survival or apoptosis. For example, the ovarian carcinoma cell line 2008, which is sensitive to cisplatin-induced apoptosis, shows sustained JNK activation and c-jun phosphorylation to at least 12 h whereas its resistant variant displays only transient activation. In HCT116 cells we observed activation of JNK to at least 24 h. It is important to note that interference with JNK signalling using the method described here did not result in total abolition of curcumin-induced cell death, as shown by both MTT and PARP cleavage assays. This may be due to incomplete inhibition of JNK by SP600125, since treatment with the inhibitor led to significant and reproducible, but not total, reduction in phosphorylation of c-jun. However, it is likely that JNK-independent mechanisms may also contribute to curcumin induction of apoptosis.

Our findings of curcumin inhibition of a constitutively activated NFκB-dependent luciferase reporter construct, accord with previous observations (47,48). Sustained activation of JNK resulting from inhibition of NFκB has been attributed to disruption of the negative modulation of JNK activation exerted by NFκB responsive genes such as XIAP (30) and gadd45β (29). Accordingly, curcumin has been shown to inhibit XIAP expression during apoptosis in melanoma cells (12). Dietary isothiocyanates are chemopreventive and induce JNK-dependent apoptotic signalling by suppressing expression of the JNK-specific phosphatase M3/6 (54). Conceivably,
curcumin could invoke similar pathways although further work is necessary to investigate the precise mechanisms responsible.

Previous work has shown that curcumin can block cytokine- and phorbol ester-stimulated JNK activation, c-jun phosphorylation and AP-1 transcriptional activity (35,55,56). On this basis, curcumin has been used as an inhibitor of JNK and AP-1. While these effects may be dose- or cell type-specific, our results, showing curcumin activation of JNK, phosphorylation of c-jun and activation of AP-1 in HCT116 cells indicate that such uses of curcumin should be approached with caution.

In conclusion, our results show that curcumin induces apoptosis in human colon cancer cells in a JNK-dependent manner. Further studies of curcumin-mediated JNK-dependent mechanisms of apoptosis in human colon cells, appear indicated.

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References


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