Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells

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As inflammation emerges as a risk factor for prostate cancer (PCa), there is potential for chemoprevention by anti-inflammatory agents. Dietary phytochemicals have been shown to have chemopreventive properties which may include anti-inflammatory activities. In this study, we demonstrate a role for mitogen-activated protein kinase phosphatase-5 (MKP5) in mediating anti-inflammatory activities of the phytochemicals curcumin, resveratrol and [6]-gingerol. We utilized the cytokine tumor necrosis factor-α (TNFα) and interleukin (IL)-1β to increase p38-dependent nuclear factor kappa-B (NFκB) activation and expression of pro-inflammatory genes cyclooxygenase-2 (COX-2), IL-6 and IL-8 in normal prostatic epithelial cells. MKP5 over-expression decreased cytokine-induced NFκB activation, COX-2, IL-6 and IL-8 in normal prostatic epithelial cells, suggesting potent anti-inflammatory activity of MKP5. Pretreatment of cells with a p38 inhibitor mimicked the results observed with MKP5 over-expression, further implicating p38 inhibition as the main activity of MKP5. Curcumin, the phytochemical found in turmeric, up-regulated MKP5, suggesting utility of these phytochemicals in PCa treatment. In prostate cells and PCa cell lines. Lastly, we found that MKP5 was also up-regulated by curcumin has been shown to have anti-inflammatory activities attributed to curcumin may be a result of MKP5-mediated anti-inflammatory activity. Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MKP5 in prostate cells. Using a pro-inflammatory cytokine stress, we demonstrated an anti-inflammatory role of MKP5 in normal prostatic epithelial cells by exogenous modulation of MKP5 levels with an expression vector and with small interfering RNA (siRNA). Curcumin up-regulated MKP5 and inhibited pro-inflammatory signaling in both normal prostatic epithelial cells and PCa cell lines. Lastly, we found that MKP5 was also upregulated by the phytochemicals resveratrol and [6]-gingerol in normal prostatic epithelial cells and PCa cell lines.

Introduction

As the carcinogenic processes that ultimately lead to prostate cancer (PCa) are being revealed, inflammation has emerged as a significant risk factor for PCa development (1). Tissue inflammation can contribute to cancer development by inducing oxidative damage and promoting cell growth (2). Inflammation has been shown to play a causal role in the initiation and/or progression of other cancers, including liver, bladder and gastric cancers (2). Evidence linking inflammation to PCa includes (i) regular administration of non-steroidal anti-inflammatory drugs significantly decreased PCa risk in older men by 60–80% (3,4); (ii) men with chronic and/or acute inflammation of the prostate have an increased risk of developing PCa (1,5) and (iii) inflammatory cytokines are over-expressed in serum and prostate tissue of PCa patients. Furthermore, Nelson et al. (1) have characterized and identified areas within the prostate of proliferative inflammatory atrophy, a possible precursor lesion to adenocarcinoma, suggesting a multi-step process of PCa development.

The long latency observed in PCa is unique among malignancies and should provide a lengthy window of opportunity for intervention by chemopreventive agents. The hope of PCa prevention by ‘natural’ dietary products, also known as phytochemicals, has become increasingly popular and concordantly the mechanism of action of phytochemicals has become an active area of research. With the accumulation of evidence linking inflammation to PCa, anti-inflammatory activities may be a significant mechanism by which phytochemicals decrease PCa risk.

We have previously characterized mitogen-activated protein kinase phosphatase-5 (MKP5) as a mediator of anti-inflammatory activities of vitamin D [1,25-dihydroxyvitamin D3, (1,25D)] in the prostate (6). Although not typically considered a phytochemical, vitamin D is an essential part of the diet and has been implicated in the prevention of PCa by epidemiologic and laboratory studies (7). cDNA microarray experiments originally identified MKP5 as a gene up-regulated by vitamin D in prostate (8), skin (9), colon (10) and ovarian cells (11). MKP5 is a member of the dual specificity MAPK family of protein kinases that dephosphorylate mitogen-activated protein kinases (MAPKs). MKP5 specifically dephosphorylates the stress-activated protein kinases p38 and jun N-terminal kinase (JNK) (12,13). The MKP5 knockout mice revealed that MKP5 regulates both innate and adaptive immune responses in vivo (14).

The ability of MKP5 to inhibit p38 is of interest to PCa prevention since p38 signaling mediates pro-inflammatory responses in cells (15), potentially contributing to PCa development (1). Downstream anti-inflammatory effects of p38 inhibition can include decreased activation of nuclear factor kappa B (NFκB), reduced cyclooxygenase-2 (COX-2) expression and decreased production of pro-inflammatory cytokines. Consistent with p38 inhibition, we found that up-regulation of MKP5 by 1,25D decreased p38 phosphorylation and subsequently reduced interleukin (IL)-6 production in prostatic epithelial cells derived from normal tissues and localized adenocarcinomas (6). 1,25D also decreased COX-2 expression in prostate cells (16) and NFκB activity (17) in melanoma cell lines, effects potentially mediated by MKP5.

The anticancer activity of curcumin (diferuloylmethane), a polyphenol from turmeric (Curcuma longa Linn.), has been extensively studied and is well established in vitro and in vivo (18). In PCa cell lines, curcumin and analogs of curcumin inhibit growth, induce apoptosis and decrease androgen receptor signaling (18). Anti-inflammatory properties of curcumin have been shown in keratinocytes in which curcumin inhibited p38 signaling and NFκB activation (19). Furthermore, Yan et al. (20) identified MKP5 as a gene up-regulated by curcumin in MDA-MB-231 squamous cell carcinoma cells. Since curcumin has been shown to have anti-inflammatory activity and inflammation may be a risk factor for PCa, some of the chemopreventive activities attributed to curcumin may be a result of MKP5-mediated anti-inflammatory activity.

The aim of these experiments was to investigate the potentially chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MKP5 in prostate cells. Using a pro-inflammatory cytokine stress, we demonstrated an anti-inflammatory role of MKP5 in normal prostatic epithelial cells by exogenous modulation of MKP5 levels with an expression vector and with small interfering RNA (siRNA). Curcumin up-regulated MKP5 and inhibited pro-inflammatory signaling in both normal prostatic epithelial cells and PCa cell lines. Lastly, we found that MKP5 was also upregulated by the phytochemicals resveratrol and [6]-gingerol in normal prostatic epithelial cells and PCa cell lines.
Materials and methods

Cell culture and reagents

Human primary prostatic epithelial cells were derived from radical prostatectomy specimens. The patients did not have prior chemical, hormonal or radiation therapy. Histological characterization of the tissue of origin and culture of the prostate cells was as described previously (21). Epithelial cells derived from the normal peripheral zone of the prostate (E-PZ) were cultured in supplemented MCDB 105 (Sigma–Aldrich, St Louis, MO) or FPMR-4A as described previously (21). Human PCa cells line LnCaP PC-3 and DU 145 were acquired from American Type Culture Collection (Manassas, VA). LnCaP cells were cultured in MCDB 105/10% fetal bovine serum, and PC-3 and DU 145 in DMEM (Invitrogen, Carlsbad, CA)/10% fetal bovine serum. LAPC-4 cells (provided by Dr Charles Sawyers, UCLA, Los Angeles, CA) were cultured in phenol red-free RPMI 1640 (Life Technologies, Rockville, MD)/10% fetal bovine serum. All chemicals were obtained from Sigma–Aldrich unless otherwise noted. SB202190 was obtained from Calbiochem (San Diego, CA), reconstituted in dimethyl sulfoxide (DMSO) and stored at −20°C. Currumin and ginger (Wako Pure Chemicals, Osaka, Japan) were reconstituted in 5% DMSO/95% ethanol and stored at −20°C. Currumin and ginger were used with 75% confluent 60 mm collagen-coated plates. In Figure 2C, 250 and 500 ng of pcDNA3.1 plasmid were included in the transfection. Eight hours after transfection, cells were treated as indicated in figure legends. Luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega). The ratio of luciferase to Renilla luciferase was determined to correct for transfection efficiency.

RNA isolation and real-time quantitative reverse transcription–polymerase chain reaction

RNA was isolated from cells by lysis in TriZOL® (Invitrogen) followed by chloroform extraction. The aqueous phase was then precipitated in 100% iso-propanol and the pellet washed in 75% ethanol before re-suspension in water. RNA concentration and quality were determined by absorbance ratio at 260/280 nm using a UV spectrophotometer. Total RNA (2 μg) was reverse transcribed using Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA). Resulting cDNA was used for quantitative polymerase chain reaction amplification with gene-specific primers and the Brilliant SYBR Green kit (Stratagene) in the MX3005-Pro thermocycler (Stratagene). Polymerase chain reaction conditions for all primer sets were optimized and have similar amplification efficiency under the following thermocycler conditions: 95°C, 5 min; 34× (95°C, 30 s; 58°C, 30 s and 72°C, 60 s) and 72°C, 5 min. Relative messenger RNA (mRNA) levels were calculated from the point where each curve crossed the threshold line (Ct) using the equation: relative value = 2^(-ΔCt) (control) – Ct (test) gene expression/ΔCt (control) – ΔCt (test) housekeeping gene (22). Reactions were performed in triplicate and the values were normalized to the expression of the housekeeping gene TATA box-binding protein (23). Primer sets were TATA box-binding protein: 5’-ctgcggaggaagctgttag-3’ and 5’-tctgattagctgtagggccc-3’; MKP5: 5’-aattggtcgtttgccttt-3’ and 5’-ggcttattacagtggcaatgag-3’; IL-8: 5’-aggcttattacagtggcaatgag-3’ and 5’-aggcggtgcttgttcctcag-3’; COX-2: 5’-acactcaggcagatgatctaccc-3’ and 5’-ctgtagtggtggtcagta-3’; TATA box-binding protein: 5’-gctgtagtggtggtcagta-3’ and 5’-tagtggtggtcagta-3’. MKP5 over-expression

Full-length MKP5 cDNA (GenBank accession no. BC063826) was cloned into pcDNA3.1 (Invitrogen) using primers 5’-taattggtcgtttgccttt-3’ and 5’-gttgaaggagctgttaggccc-3’ and confirmed by sequence analysis. pcDNA3.1-MKP5 or pcDNA3.1-LacZ were transiently transfected into 50% confluent E-PZ cells using siPORT NeoFX transfection reagent (Ambion). In Figure 2A, B and D, 1 μg of plasmid DNA and 7 μl of siPORT NeoFX transfection reagent were used with 75% confluent 60 mm collagen-coated dishes.

NFκB-luciferase reporter activity assay

NFκB-luciferase construct (Clontech, Mountain View, CA) containing four tandem copies of the NFκB consensus sequence driving luciferase expression and pRL-null-Renilla (Promega, Madison, WI) were transiently transfected into E-PZ cells using siPORT NeoFX reagent. Cells (4.5 × 10^4 cells per well) were transfected in suspension with 10 ng of plasmid and 1 μl of NeoFX and plated onto 24-well collagen-coated plates. In Figure 2C, 250 and 500 ng of pcDNA3.1 plasmid were included in the transfection. Eight hours after transfection, cells were treated as indicated in figure legends. Luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega). The ratio of luciferase to Renilla luciferase was determined to correct for transfection efficiency.

Results

Characterization of TNFα and IL-1β as inducers of p38-dependent NFκB activation, COX-2 expression and pro-inflammatory cytokine production in normal prostatic E-PZ cells

To evaluate the role of MKP5 in the inflammatory response of E-PZ, it was necessary to identify a pro-inflammatory stimulus that signaled through p38, TNFα and IL-1β are potent pro-inflammatory cytokines secreted by macrophages to initiate inflammation by stimulating the expression of pro-inflammatory genes such as IL-6, IL-8 and COX-2 (24). The pro-inflammatory cytokines IL-6 and IL-8 are of particular interest to PCa prevention because they are characteristically over-expressed in PCA tissue and patient serum (25). COX-2 functions to increase prostaglandin secretion from prostate cells, which can further facilitate inflammatory cell recruitment as well as increase cell proliferation (16). IL-1β and TNFα also induce their own expression causing an amplification loop of the inflammatory response (24). Importantly, TNFα and IL-1β have been shown to signal through both the p38 MAPK and the NFκB pathways (24,26).

E-PZ cells were stimulated with a combination of 1 ng/ml TNFα and 5 ng/ml IL-1β (T+1) for 15 min and 1, 3, 6 and 24 h. Cells were co-treated because TNFα and IL-1β showed a synergistic effect on p38 phosphorylation, COX-2 and inflammatory cytokine expression in the prostate cells (data not shown), which is similar to other published results (27,28). Cell lysates and RNAs were collected at each time point. Immunoblot of cell lysates showed that phosphorylation of p38 occurred within 15 min of T+1 treatment (Figure 1A). COX-2 mRNA and protein was increased after 6 h of T+1 (Figure 1A and B). JNK phosphorylation was not stimulated by T+1 in the E-PZ cells (data not shown). Since NFκB has been implicated in both TNFα and IL-1β signaling, NFκB activation by T+1 was determined by luciferase enzyme activity of a transiently transfected NFκB-luc reporter construct. Twenty-four hours following transfection with NFκB-luc and pRL-null, E-PZ cells were treated with T+1 for 8 h and luciferase activity was measured. T+1 stimulation increased NFκB-luc activity 10-fold (Figure 1C). NFκB p65 nuclear translocation and activation were dependent upon phosphorylation and subsequent degradation of the inhibitory protein IκBα (29). This is a point at which the p38 and NFκB pathways converge via p38-mediated phosphorylation of IκBα (30). To test whether T+1-stimulated NFκB activity was dependent upon p38 signaling in the E-PZ cells, SB202190, a small molecule inhibitor of p38, was used. The results showed that in E-PZ cells, T+1-stimulated NFκB activity was dependent upon p38 signaling as 1 h pretreatment with 10 μM SB202190 completely blocked the increase in NFκB-luc activity (Figure 1C).

Increased mRNA accumulation of the pro-inflammatory cytokines IL-6, IL-8, IL-1β and TNFα can occur by two mechanisms as follows: (1) increased p38-mediated mRNA stability and protein translation
(31) and (ii) increased NFκB-mediated gene transcription (32). In E-PZ cells, we observed increased mRNA accumulation of IL-6, IL-8, IL-1β and TNFα at 1 h following T+I with maximum mRNA levels at 6 h (Figure 1D). Levels of IL-1β and TNFα mRNA were measured as positive controls, because IL-1β and TNFα stimulate their own gene expression (24). SB202190 was used to test whether cytokine mRNA stabilization was dependent upon p38 signaling. A 1 h pre-treatment with 10 μM SB202190 completely blocked the increase in cytokine mRNA by T+I (Figure 1E). MKP5 mRNA levels were unchanged by T+I (data not shown).

Therefore, T+I stimulation was used in all subsequent experiments because it caused a robust increase in cytokine mRNA, COX-2 mRNA and protein, and NFκB activity in E-PZ cells, all of which could be blocked by SB202190 and hence were p38 dependent.

**MKP5 inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 expression and cytokine production in E-PZ cells**

Our next goal was to assess whether MKP5, as a p38 MKP, could inhibit or decrease the powerful pro-inflammatory molecular changes induced by T+I stimulation in E-PZ cells. We showed previously that up-regulation of MKP5 by 1,25D attenuated UV irradiation- and TNFα-induced p38 activation and IL-6 secretion in E-PZ cells (6).

To determine the role of MKP5 in the T+I response, the intracellular levels of MKP5 were up-regulated by an expression plasmid and down-regulated with siRNA. E-PZ cells were transiently transfected with pcDNA3.1-LacZ or pcDNA3.1-MKP5. Transfection with pcDNA3.1-MKP5 increased MKP5 mRNA levels 10- to 50-fold at 24 h (data not shown). Twenty-four hours following transfection, cells were stimulated with T+I for 15 min and p38 phosphorylation was measured by immunoblot of cell lysates. The cells transfected with pcDNA3.1-MKP5 had attenuated levels of phospho-p38 compared with pcDNA3.1-LacZ, showing that the exogenous MKP5 was active (Figure 2A). Exogenous MKP5 was able to block T+I-stimulated COX-2 expression as pcDNA3.1-MKP5-transfected E-PZ cells showed less COX-2 protein by immunoblot analysis (Figure 2B). As a control, SB202190 was used and blocked basal and T+I-stimulated COX-2 protein expression (Figure 2B). Conversely, when MKP5 levels were decreased in E-PZ cells by siRNA, basal and T+I-induced COX-2 protein expression was increased compared with negative control siRNA (Figure 2B). MKP5-siRNA decreased MKP5 mRNA levels to 20% of NEG-siRNA (data shown in Figure 3C). Since T+I-induced NFκB activation was dependent upon p38 signaling, the effect of exogenous MKP5 on NFκB-luc was measured. The luciferase assay showed that transfection of E-PZ cells with pcDNA3.1-MKP5 inhibited NFκB-luc activity in a dose-dependent manner (Figure 2C). Consistent with decreased p38 signaling and NFκB activity, the levels of T+I-induced IL-6 and IL-8 were reduced >10-fold in E-PZ cells transfected with pcDNA3.1-MKP5 compared with control vector (Figure 2D).

These results reveal that MKP5 has a significant role in reducing T+I-stimulated inflammatory signaling by inhibiting p38-mediated pro-inflammatory processes in normal prostatic E-PZ cells.
MKP5 was up-regulated by curcumin and inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 expression and cytokine production in E-PZ cells.

Curcumin is the polyphenolic compound present in the spice turmeric. Curcumin has been shown to have significant anti-inflammatory effects in a variety of cell types (19). Interestingly, curcumin was found to up-regulate MKP5 mRNA in an MDA-1986 squamous cell carcinoma microarray study (20). We speculated that curcumin would have similar anti-inflammatory effects in the normal prostatic E-PZ cells and that these effects would be mediated by MKP5.

In agreement with our hypothesis, curcumin dose dependently up-regulated MKP5 mRNA (Figure 3A) and 17 h pretreatment with 50 μM curcumin inhibited T + I-stimulated p38 phosphorylation in E-PZ cells (Figure 3B). Curcumin did not affect mRNA stability of MKP5 as mRNA half-life following actinomycin D treatment was not different between vehicle- and curcumin-treated cells (L.Nonn, unpublished results). Curcumin pretreatment also blocked T + I-stimulated COX-2 protein expression and the effect was partially attenuated by MKP5-siRNA (Figure 3C). MKP5-siRNA attenuated MKP5 up-regulation by curcumin to 20% of NEG-siRNA (Figure 3C). Downstream of p38 signaling by T + I, NFκB-luc activity and cytokine mRNA accumulation were blocked by 17 h pretreatment with 50 μM curcumin (Figure 3D and E).

These results show that MKP5 is up-regulated by curcumin and mediates anti-inflammatory activities of curcumin in normal prostatic E-PZ cells.

**PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain ability to up-regulate MKP5 in response to curcumin**

In our previous study, we found that 1,25D up-regulated MKP5 only in primary E-PZ cells and cells cultured from primary adenocarcinomas of the prostate, but not in the PCa cell lines DU 145, PC-3 and LNCaP (6). 1,25D typically alters gene expression through binding to the vitamin D receptor (VDR). VDR is a classical steroid receptor that translocates to the nucleus and binds to vitamin D response elements in the promoter regions of genes. This is the likely mechanism for regulation of MKP5 by 1,25D because the MKP5 promoter contains a putative vitamin D response element and up-regulation of MKP5 by 1,25D was dependent upon VDR (6). We did not find that curcumin up-regulated any of the classic VDR-regulated genes (data not shown) and this photochemical has not been reported to alter gene expression through VDR activation. The mechanism for MKP5 up-regulation by curcumin is probably distinct from that of 1,25D, and therefore MKP5 regulation by curcumin was examined in the immortal PCa cell lines DU 145, PC-3, LNCaP and LAPC-4.

It is well established that curcumin can induce cell death in PCa cells at high concentrations (33). After initially testing a range of curcumin concentrations (1–50 μM), we found that 25 μM curcumin was able to significantly up-regulate MKP5 mRNA in DU 145, PC-3, LNCaP and LAPC-4 cells (Figure 4A) without causing visible cell death [consistent with published data (33) therefore not shown]. When stimulated with T + I, only the DU 145 cells responded with increased p38 phosphorylation and COX-2 expression. In the DU 145 cells, pretreatment with 25 μM curcumin was able to block T + I-induced p38 phosphorylation and COX-2 protein expression (Figure 4B). Pretreatment of DU 145 cells with curcumin decreased COX-2 mRNA similarly to SB202190 (Figure 4B), showing COX-2 expression was p38 dependent. T + I-induced NFκB-luc activity was not decreased by pretreatment of DU 145 cells with either 10 μM SB202190 or 25 μM curcumin (Figure 4C). This finding correlates with published data showing that DU 145 cells have constitutively elevated NFκB activity due to increased activity of IκB kinase (34), which is p38 independent (30). However, IL-6 and IL-8 cytokine mRNA levels were decreased.
by curcumin or SB202190 pretreatment (Figure 4D), suggesting that T + I-induced expression of IL-6 and IL-8 is mediated by p38 rather than NFκB in DU 145 cells.

These results show that the PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain the ability to up-regulate MKP5 in response to curcumin and that curcumin reduced p38-mediated, but not NFκB-mediated, pro-inflammatory signaling by T + I in DU 145 cells.

**MKP5 is up-regulated by the phytochemicals resveratrol and [6]-gingerol in normal prostatic E-PZ cells and PCa cell lines**

Two other phytochemicals, resveratrol and [6]-gingerol, have been shown to inhibit p38 phosphorylation and NFκB activation in mouse skin (35,36). Since MKP5 was up-regulated by 1,25D and curcumin, by presumably different mechanisms, we wondered if MKP5 could be up-regulated and play a role in the anti-inflammatory activities of resveratrol and [6]-gingerol in the prostate.

The ability of resveratrol and [6]-gingerol to regulate MKP5 expression in normal prostatic E-PZ cells and in PCa cell lines was determined. In E-PZ cells, we found that both 50 μM resveratrol and 50 μM [6]-gingerol increased MKP5 mRNA to levels similar to those induced by 1,25D and curcumin (Figure 5A). With the exception of resveratrol in LNCaP cells, 50 μM resveratrol or 50 μM [6]-gingerol was also able to up-regulate MKP5 in the PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 (Figure 5B).

These findings show that MKP5 is a common target of 1,25D and the phytochemicals curcumin, resveratrol and [6]-gingerol in normal prostatic E-PZ cells, whereas in the PCa cell lines, MKP5 is up-regulated by curcumin, resveratrol and [6]-gingerol but not by 1,25D.

**Discussion**

We previously identified MKP5 as a mediator of inhibition of p38 and suppression of IL-6 by 1,25D in normal prostatic epithelial cells (6). The current study expands on the inhibitory role of MKP5 in pro-inflammatory pathways in prostatic cells. Here, a combination of T + I was chosen as the pro-inflammatory stimulus in E-PZ cells because it rapidly increased COX-2 protein levels, NFκB activity and cytokine mRNA accumulation via a p38-dependent pathway. NFκB activation was not involved in T + I-stimulated inflammatory signaling as JNK phosphorylation was not detected. TNFα and IL-1β are also physiologically relevant to prostatic inflammation since they are secreted by resident macrophages and function as ‘alarm cytokines’ to initiate inflammatory cell recruitment by stimulating the expression of pro-inflammatory genes (24) (Figure 6A).

We focused on T + I-induced expression of the pro-inflammatory genes for COX-2 and the cytokines IL-6 and IL-8. COX-2 is the inducible form of COX, the rate-limiting enzyme in prostaglandin synthesis (16). COX-2 expression results in prostaglandin secretion, which can increase cell proliferation and inflammatory cell recruitment (16). The precise nature of COX-2 expression changes during Ca development and progression remain under debate. However, high COX-2 levels at radical prostatectomy are an independent predictor of Ca recurrence (37). IL-6 and IL-8 are both found to be over-expressed in the tissues and serum of Ca patients (25,38,39). Elevated serum levels of IL-6 are associated with aggressive pathology and decreased survival of Ca patients (40). IL-8 has pro-angiogenic activity and is expressed by prostatic epithelial cells (41). In vitro, all the Ca-derived cell lines DU 145, PC-3 and LNCaP express the IL-6 receptor and are responsive to exogenous IL-6. However, DU 145 and

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Fig. 3. MKP5 was up-regulated by curcumin and inhibited TNFα- and IL-1β-stimulated p38 phosphorylation, COX-2 up-regulation, NFκB activation and cytokine production in E-PZ cells. (A) Real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) measurement of MKP5 mRNA in E-PZ cells 17 h after treatment with vehicle (0.01% EtOH), 10 and 50 μM curcumin. (B) Immunoblot of E-PZ cell lysates 30 min following T + I treatment. Blot was probed with antibodies to phospho-p38 and total p38. (C) Immunoblot of E-PZ cell lysates 24 h after T + I treatment in cells pretreated with vehicle (0.01% EtOH) or 50 μM curcumin. Four hours prior to pretreatment, cells were transfected with 10 nmol of siRNA-NEG or siRNA-MKP5. Blot was probed with antibodies to COX-2 and actin. qRT–PCR measurement of MKP5 mRNA to show knock-down with siRNA-MKP5 relative to siRNA-NEG. (D) Transactivation of NFκB-luc reporter construct in E-PZ cells, pretreated for 17 h with vehicle (0.01% EtOH), 10 and 50 μM curcumin, 8 h following T + I treatment. Data are representative of two independent experiments, and error bars represent standard deviation of triplicate samples. (E) Real-time qRT–PCR measurement of IL-6 and IL-8 mRNA after 3 h of T + I treatment in E-PZ cells, pretreated for 17 h with vehicle (0.01% EtOH) or 50 μM curcumin. Dashed line represents basal mRNA levels for cytokines. For real-time qRT–PCR experiments, mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TATA box-binding protein. Data are representative of three independent experiments, and error bars represent standard deviation of duplicate or triplicate samples.
PC-3 also constitutively over-express IL-6 whereas the LNCaP cell line does not express any IL-6 (39). Monoclonal antibody inhibition of IL-6 in LuCaP xenograft-bearing mice blocked conversion to androgen independence following castration (42). Therefore, inhibition of IL-6 may prove to be a powerful tool in PCa treatment.

We showed that when MKP5 levels were increased in normal prostatic E-PZ cells by an MKP5 expression plasmid, pro-inflammatory effects of T + I stimulation were diminished. MKP5 decreased the levels of phosphorylated p38 protein, COX-2 protein, NFκB activation and cytokine (IL-6, IL-8, TNFα and IL-1β) mRNA accumulation.

Fig. 4. PCa cell lines DU 145, PC-3, LNCaP and LAPC-4 retain ability to up-regulate MKP5 in response to curcumin. (A) Real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) measurement of MKP5 mRNA in DU 145, PC-3, LNCaP and LAPC-4 following 17 h treatment with vehicle (0.01% EtOH) or 25 μM curcumin. (B) Immunoblot of DU 145 cell lysates (10 μg) following 30 min and 24 h of T + I treatment. Blots were probed with antibodies to phospho-p38, total p38, COX-2 and actin. Real-time qRT–PCR measurement of COX-2 mRNA after 6 h of T + I treatment in DU 145 cells, pretreated for 17 h with vehicle (0.01% DMSO) or 25 μM curcumin or 1 h with 10 μM SB202190. (C) Transactivation of NFκB-luc reporter construct in DU 145 cells 8 h following T + I treatment. Cells were pretreated for either 17 h with vehicle (0.01% EtOH) or 25 μM curcumin or 1 h with 10 μM SB202190. Data are representative of two independent experiments, and error bars represent standard deviation of triplicate samples. (D) Real-time qRT–PCR measurement of IL-6 and IL-8 mRNA after 3 h of T + I treatment in DU 145 cells, pretreated for 17 h with vehicle (0.01% EtOH) or 25 μM curcumin or 1 h with 10 μM SB202190. For qRT–PCR experiments, mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TATA box-binding protein. Data are representative of three independent experiments, and error bars represent standard deviation of duplicate or triplicate samples.

Fig. 5. MKP5 was up-regulated by the phytochemicals resveratrol and gingerol in normal prostatic E-PZ cells and PCa cell lines. (A) Real-time quantitative reverse transcription–polymerase chain reaction (qRT–PCR) measurement of MKP5 mRNA in E-PZ cells following 17 h treatment with vehicle (0.01% EtOH), 50 nM 1,25D, 50 μM curcumin, 25 μM resveratrol or 25 μM gingerol. (B) Real-time qRT–PCR measurement of MKP5 mRNA in DU 145, PC-3, LNCaP and LAPC-4 following 17 h treatment with vehicle (0.01% EtOH), 25 μM curcumin, 25 μM resveratrol or 25 μM gingerol. For real-time qRT–PCR experiments, mRNA levels are shown relative to untreated control and normalized to expression of the housekeeping gene TATA box-binding protein. Data are representative of three independent experiments, and error bars represent standard deviation of duplicate or triplicate samples.
Pretreatment of the cells with SB202190, a small molecule inhibitor of p38, mimicked the anti-inflammatory effects of MKP5 expression and verified p38 dependence of T + I-induced COX-2, NFκB activation and cytokine mRNA. Conversely, when levels of MKP5 were decreased by siRNA, the pro-inflammatory effects of T + I were exaggerated. These results reveal that MKP5 has a significant role in reducing T + I-stimulated inflammatory signaling in normal prostatic epithelial cells.

Curcumin, the phytochemical present in turmeric that is believed to have cancer preventive or therapeutic capabilities, had been shown to up-regulate MKP5 mRNA in MDA-1986 squamous carcinoma cells (20). We showed that, as in MDA cells, curcumin was able to up-regulate MKP5 mRNA and inhibit p38 phosphorylation in normal prostatic E-PZ cells. Concordantly, the T + I-stimulated pro-inflammatory response in E-PZ cells was blunted by curcumin pretreatment. When siRNA was used to block the up-regulation of MKP5 by curcumin, COX-2 suppression by curcumin was attenuated, further implicating MKP5 as a mediator of curcumin’s activity. T + I-stimulated NFκB activity was also blocked by curcumin. NFκB is a pro-inflammatory transcription factor whose regulation can be complex, involving many pathways. In the E-PZ cells, NFκB activation by T + I was completely p38 dependent and thus was inhibited by curcumin via MKP5. P38-dependent NFκB activation is not unique to the stimulus that we used, but also occurs during UV irradiation and is IkB kinase independent (30). These results show that MKP5 mediates anti-inflammatory activities of curcumin in normal prostatic E-PZ cells.

In our previous study, we observed that up-regulation of MKP5 by 1,25D was unique to prostate cells derived from normal tissues or localized adenocarcinomas (6). In the metastases-derived PCa cell lines, MKP5 basal levels were lower and were unchanged by 1,25D treatment (6). In our current study, we found that curcumin, in contrast to 1,25D, up-regulated MKP5 in normal prostatic E-PZ cells as well as in the metastases-derived PCa cell lines DU 145, PC-3, LNCaP and LAPC-4. In order to explore the relevance of induction of MKP5 to anti-inflammatory activities of curcumin in the PCa cell lines, we evaluated the response of the cell lines to T + I. Stimulation of the PCa cell lines by T + I selectively stimulated p38 phosphorylation only in DU 145 cells. This was not unanticipated since both TNFa and IL-1b signal through receptor-mediated pathways that do not necessarily involve p38 MAPK (30). In the DU 145 cells, curcumin decreased T + I-stimulated p38 phosphorylation, COX-2 protein expression and IL-6 and IL-8 mRNA levels. Although DU 145 cells have constitutively activated NFκB (43), we found that T + I treatment further increased NFκB activity. However, activation of NFκB by T + I in the DU 145 cells, in contrast to E-PZ cells, was p38 independent as it was not blocked by curcumin or SB202190. Thus, although MKP5 is up-regulated by curcumin in the PCa cell lines, its activity is limited to p38-mediated effects on cytokine and COX-2 expression as NFκB activity is p38 independent in those cells.

Given the involvement of MKP5 in anti-inflammatory activities of two chemopreventive compounds, vitamin D and curcumin, we investigated several other phytochemicals suggested to have anticancer properties. Resveratrol (trans-3,5,4’-trihydroxystilbene) is a polyphenol present in grape skin and red wine (44). Jang et al. (44) have demonstrated chemopreventive activity of resveratrol in the initiation, promotion and progression stages of carcinogenesis. In PCa cell lines, resveratrol inhibits cell growth and decreases androgen receptor signaling (45). [6]-Gingerol, the major pungent phenolic found in ginger (Zingiber officinale Roscoe, Zingiberaceae), has been utilized extensively in oriental medicine for alleviation of inflammation and gastrointestinal ailments (46). However, prostate-specific anticancer activities of [6]-gingerol have not yet been studied. Both resveratrol and [6]-gingerol inhibit phorbol ester-induced COX-2 expression and NFκB activation (35,36), activities we have shown can be mediated by MKP5. We found that resveratrol and [6]-gingerol, like vitamin D and curcumin, up-regulated MKP5 in normal prostatic E-PZ cells and in the PCa cell lines, with the exception of resveratrol in the LNCaP cells. In general, we found that LNCaP cells had limited ability to up-regulate MKP5 in response to any of the phytochemicals, suggesting the presence of genomic or epigenomic suppression. MKP5 mRNA up-regulation by 1,25D and the phytochemicals is presumably via different mechanisms, as 1,25D altered MKP5 gene expression through the VDR (6) and the other phytochemicals do not. Although our studies focused on mRNA regulation of MKP5, the possibility of further post-translational regulation of MKP5 activity cannot be excluded. Since MKP5 is a common target of these and potentially other phytochemicals, it is possible that combination dosing strategies could amplify MKP5 up-regulation, perhaps increasing anti-inflammatory activities while decreasing dosage of individual phytochemicals, thus decreasing side effects.

The fact that the PCa cell lines retain the ability to up-regulate MKP5 in response to phytochemicals is relevant to PCa therapy. It shows that not only can phytochemicals ingested in the diet and via supplements play a role in PCa prevention but also that phytochemicals can be exploited for use in PCa treatment. P38 MAPK has diverse biological properties and activation can mediate apoptosis or cell survival, depending upon the type of stress, cell background and activities of the other MAPKs (15). Therefore, whereas inhibition of the p38 pathway in normal tissue is an anti-inflammatory chemopreventive activity, p38 inhibition in PCa could affect cell survival...
pathways leading to increased sensitivity to chemotherapies. In fact, all the phytochemicals we analyzed are currently under heavy investigation for their utility in cancer treatment and analogs with enhanced antitumor activity are being tested.

In conclusion, these experiments show that (i) MKP5 is a potent inhibitor of pro-inflammatory signaling in prostate cells and (ii) MKP5 may be a common mediator of phytochemical anti-inflammatory activities. Our data suggest that, in the prostate, TNF-α and IL-1β-induced inflammatory cell recruitment could theoretically be decreased by phytochemical-induced MKP5 (Figure 6B), leading to a reduction in prostate inflammation. Also, in contrast to 1,25D, which does not up-regulate MKP5 in PCa cell lines, curcumin, resveratrol and ginseng were able to up-regulate MKP5 and inhibit the p38 pathway in the PCa cell lines, implicating potential utility in management of early or advanced PCa. Although we believe MKP5 to be an important mediator of inflammation in prostate cells, we are not downplaying other molecular effects of the phytochemicals. Ultimately, phytochemicals have the ability to affect many pathways and a greater understanding of their mechanisms of action will facilitate exploitation of these naturally occurring ‘drugs’ for cancer prevention and therapy.

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References


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