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RESEARCH PAPER

Curcumin inhibits connective tissue growth factor gene expression in activated hepatic stellate cells *in vitro* by blocking NF-κB and ERK signalling

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Background and purpose: Gene expression of connective tissue growth factor (CTGF) is induced in activated hepatic stellate cells (HSC), the major effectors in hepatic fibrosis, and production of extracellular matrix (ECM) is consequently increased. We previously reported that curcumin, the yellow pigment in curry, suppressed *ctgf* expression, leading to decreased production of ECM by HSC. The purpose of this study is to evaluate signal transduction pathways involved in the curcumin suppression of *ctgf* expression in HSC.
Experimental approaches: Transient transfection assays were performed to evaluate effects of activation of signalling pathways on the *ctgf* promoter activity. Real-time PCR and Western blotting analyses were conducted to determine expression of genes.
Results: Suppression of *ctgf* expression by curcumin was dose-dependently reversed by lipopolysaccharide (LPS), an NF-κB activator. LPS increased the abundance of CTGF and type I collagen in HSC *in vitro*. Activation of NF-κB by dominant active IκB kinase (IKK), or inhibition of NF-κB by dominant negative IκBα, caused the stimulation, or suppression of the *ctgf* promoter activity, respectively. Curcumin suppressed gene expression of Toll-like receptor-4, leading to the inhibition of NF-κB. On the other hand, interruption of ERK signalling by inhibitors or dominant negative ERK, like curcumin, reduced NF-κB activity and in *ctgf* expression. In contrast, the stimulation of ERK signalling by constitutively active ERK prevented the inhibitory effects of curcumin.
Conclusions and implications: These results demonstrate that the interruption of NF-κB and ERK signalling by curcumin results in the suppression of *ctgf* expression in activated HSC *in vitro*.
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Keywords: gene expression; hepatic fibrosis; hepatic stellate cell; polyphenol; signal transduction

Abbreviations: CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; PPARγ, peroxisome proliferator-activated receptor gamma; TGF-β, transforming growth factor-beta; TLR4, Toll-like receptor 4

Introduction

Hepatic stellate cells (HSCs), previously termed as fat- or vitamin A-storing cells, or Ito cells, are the effectors in hepatic fibrogenesis (Friedman, 2004; Kisseleva and Brenner, 2006). HSCs are quiescent and non-proliferative in the normal liver. Upon liver injury, quiescent HSCs become active, which is characterized by enhanced cell growth and overproduction of extracellular matrix (ECM) components. Culturing quiescent HSC on plastic plates causes spontaneous activation, mimicking the process seen *in vivo*, which provides a good model for elucidating underlying mechanisms of HSC activation and for studying possible therapeutic interventions in the process (Friedman, 2004; Kisseleva and Brenner, 2006). Although the causal relationship remains unclear, it has been demonstrated that activation of HSC is closely associated with activation of the transcription factor nuclear factor kappa B (NF-κB) (Hellerbrand *et al*., 1998; Rippe *et al*., 1999). Inhibitor-κB (IκB) binds NF-κB and inhibits its activation by preventing NF-κB from translocating to the nucleus. Upon activation of NF-κB, IκBα is phosphorylated by IκB kinase (IKK), leading to the dissociation of IκBα from NF-κB and the subsequent degradation of IκBα.

There is a growing body of evidence that upregulation of connective tissue growth factor (CTGF/CCN2) might be a

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central pathway during HSC activation and hepatic fibrogenesis (Paradis *et al*., 1999; Williams *et al*., 2000). CTGF gene expression in HSC is significantly enhanced during the process of activation *in vitro* and *in vivo* (Williams *et al*., 2000). HSCs are the major cellular source of CTGF in the liver during hepatic fibrogenesis (Paradis *et al*., 2002). Gene expression of type I collagen is significantly induced by transforming growth factor-beta (TGF-β) during HSC activation. CTGF is transcriptionally regulated by TGF-β and plays a key role in the overproduction of ECM in activated HSC (Fu *et al*., 2001; Blom *et al*., 2002; Leask *et al*., 2003). Blockade of CTGF synthesis by antisense oligonucleotides of CTGF resulted in down expression of type I collagen mRNA in an animal model with experimental liver fibrosis (Uchio *et al*., 2004). The level of type I collagen has been used as a downstream target and a marker for evaluating the activity/expression of CTGF in cells (Kobayashi *et al*., 2005; Xiao *et al*., 2006).

Curcumin is the main yellow pigment in curry from turmeric. Besides its dietary use, curcumin has been used as an anti-inflammatory remedy in Chinese herbal medicine for skin and intestinal diseases and for wound healing. Recent studies have indicated that dietary administration of curcumin improves both acute and subacute rat liver injury caused by carbon tetrachloride (Park *et al*., 2000). We demonstrated previously that curcumin inhibited activation of HSC *in vitro* by inhibiting cell growth and suppressing production of ECM components (Xu *et al*., 2003). Curcumin induced gene expression of the peroxisome proliferator-activated receptor gamma (PPARγ) and stimulated PPARγ signalling in activated HSC, which was a prerequisite for curcumin to inhibit HSC activation (Xu *et al*., 2003; Zheng and Chen, 2004). Our further studies demonstrated that curcumin suppressed gene expression of CTGF in activated HSC *in vitro*, leading to the reduction in the production of ECM components, including type I collagen and fibronectin (Zheng and Chen, 2006). The purpose of the present study was to investigate signal transduction pathways involved in the curcumin suppression of CTGF gene expression in activated HSC. Results presented in this study supported our hypothesis and demonstrated that the interruption of NF-κB and ERK signalling pathways by curcumin resulted in the suppression of CTGF gene expression in activated HSC. These results provide novel insights into the mechanisms of curcumin in the inhibition of HSC activation.

Materials and methods

Isolation and culture of HSCs

HSCs were isolated from male Sprague–Dawley rats (~200–250 g) as described previously (Chen and Davis, 2000). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), unless otherwise indicated. HSCs between passages 4–8 were used for experiments. Curcumin cytotoxicity was evaluated previously (Xu *et al*., 2003). It was concluded that curcumin up to 100 μM was not toxic to cultured HSC. Curcumin at 20 μM was used in this study, unless otherwise indicated.

Western blotting analyses

These were performed as described previously (Xu *et al*., 2003). In brief, whole-cell lysates were prepared using radioimmunoprecipitation analyses buffer supplemented with protease inhibitors. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. Target proteins were detected by primary antibodies against CTGF (1:1000), αI(I) pro-collagen (1:500) or Toll-like receptor 4 (TLR4) (1:1000), and subsequently by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized by using a chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ, USA).

RNA isolation and real-time PCR

Total RNA was extracted using TRI reagent (Sigma, St Louis, MO, USA) according to the protocol provided by the manufacturer. Real-time PCR was carried out using SYBR Green as described previously (Chen *et al*., 2002; Fu *et al*., 2006). mRNA fold changes of target genes relative to the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control were calculated as suggested by Schmittgen *et al*. (2000). The following primers were used for real-time PCR: CTGF: (F) 5′-TGTGTGATGAGCCCAAGGAC-3′, (R) 5′-AGTTGGCTCGCATCATAGTTG-3′; TLR_4: (F) 5′-TGGATACGT TTCCTTATAAG-3′, (R) 5′-GAAATGGAGGCACCCCTTC-3′; αI(I) collagen: (F) 5′-CCTCAAGGGCTCCAACGAG-3′, (R) 5′-TCAATCACTGTCTTGCCCCA-3′; GAPDH: (F) 5′-GGCAAATT CAACGGCACAGT-3′, (R) 5′-AGATGGTGATGGGCTTCCC-3′.

Plasmid constructs and transient transfection assays

The *ctgf* promoter luciferase reporter plasmid pCTGF-Luc, a gift from Dr Yuqing E Chen, (Cardiovascular Research Institute, Morehouse School of Medicine, Atlanta, GE, USA), contains a fragment of the *ctgf* promoter (~2000 bp nucleotides) subcloned into the luciferase reporter plasmid pGL3 (Fu *et al*., 2001). The NF-κB activity reporter plasmid pNF-κB-Luc was purchased from Clontech (Mountain View, CA, USA). The plasmids pCMV-IKK-2 S177E/S181E and pCMV-IKK-2-WT were purchased from Addgene Inc. (Cambridge, MA, USA). These two plasmids express the constitutively active form of IKK2 and wild-type IKK2, respectively. The plasmids pCMV-IκBα-WT, expressing wild-type IκBα, and pCMV-IκBα-M, encoding the dominant-negative form of IκBα (dn-IκBα), were purchased from Clontech. The plasmid pCMV-IκBα-M contains two serine to alanine mutations at residues 32 and 36, which prevents the phosphorylation of IκBα, leading to the blockade of NF-κB activation (Brown *et al*., 1995). The PPARγ cDNA-expressing plasmid pPPARγcDNA, containing a full size of PPARγ cDNA, was a gift from Dr Reed Graves (Department of Medicine, the University of Chicago, Chicago, IL, USA). The plasmid pdn-ERK contains a full length of cDNA encoding the dominant-negative form of ERK. The plasmid pa-ERK contains cDNA encoding the constitutively active form of ERK (a-ERK). Both the plasmids were described previously (Davis *et al*., 1996). Transient transfection was conducted using LipofectAMINE (Life Technologies) following the

protocol provided by the manufacturer. In brief, semiconfluent HSC in six-well culture plates were transiently transfected with reporter plasmids (~3–4 μg DNA per well). Transfection efficiency was controlled by co-transfection of the β-galactosidase reporter plasmid pSV-β (~0.5–1 μg per well) (Promega Corp.). Luciferase activity was measured using an automated luminometer (Turners). β-Galactosidase assays were performed using an assay kit from Promega Corp. Each treatment was carried out in triplicate, in every experiment. Each experiment was repeated for at least three times. Luciferase activity was expressed as relative unit after normalization with β-galactosidase activity.

Statistical analysis

Differences between means were evaluated using an unpaired two-sided Student's *t*-test ($P<0.05$ considered as significant). Where appropriate, comparisons of multiple treatment conditions with controls were analysed by ANOVA with the Dunnett's test for post hoc analysis.

Materials

Curcumin (purity > 94%), bacterial lipopolysaccharide (LPS) from *E. coli* and pyrrolidine dithiocarbamate were purchased from Sigma. PD68235, a specific PPARγ antagonist, was kindly provided by Pfizer (Ann Arbor, MI, USA) (Camp *et al*., 2001).

Results

LPS dose-dependently abolishes the inhibitory effect of curcumin on the CTGF gene promoter and induces gene expression of CTGF and αI(I) collagen in activated HSC in vitro

Curcumin suppressed the *ctgf* promoter activity and reduced CTGF protein in HSC (Zheng and Chen, 2006). Curcumin also decreased the activity of NF-κB (Xu *et al*., 2003) in these cells. As bacterial LPS is an activator of NF-κB (Hacker and Karin, 2006), we hypothesized that the effects of curcumin on NF-κB and CTGF gene expression in our model might be antagonized by exposure to LPS.

Our pilot experiments confirmed that LPS dose-dependently increased the transactivation activity of NF-κB in cultured HSC (data not shown) and that curcumin reduced the abundance of CTGF and its downstream target αI(I)procollagen in cultured HSC (inset in Figure 1a). To test our hypothesis, passaged HSCs were transiently transfected with the *ctgf* promoter luciferase reporter plasmid pCTGF-Luc, containing a fragment of the CTGF gene promoter (~2000 bp nucleotides) (Fu *et al*., 2001). After recovery, cells were treated with curcumin in the presence and absence of LPS at indicated concentrations for 24 h. As shown by luciferase assays in Figure 1a, curcumin, as expected, significantly reduced the *ctgf* promoter activity in these cells (the second column on the left), compared with that in the untreated control (the first column on the left). The inhibitory effect of curcumin was partially reversed by LPS exposure in a dose-dependent manner. Further experiments demonstrated that LPS increased the levels of the transcript and protein of CTGF and αI(I) procollagen in cultured HSCs, as shown by real-time PCR (Figure 1b) and western blotting analyses (Figure 1c), respectively. Taken together, these results suggested that the activation of NF-κB by LPS might

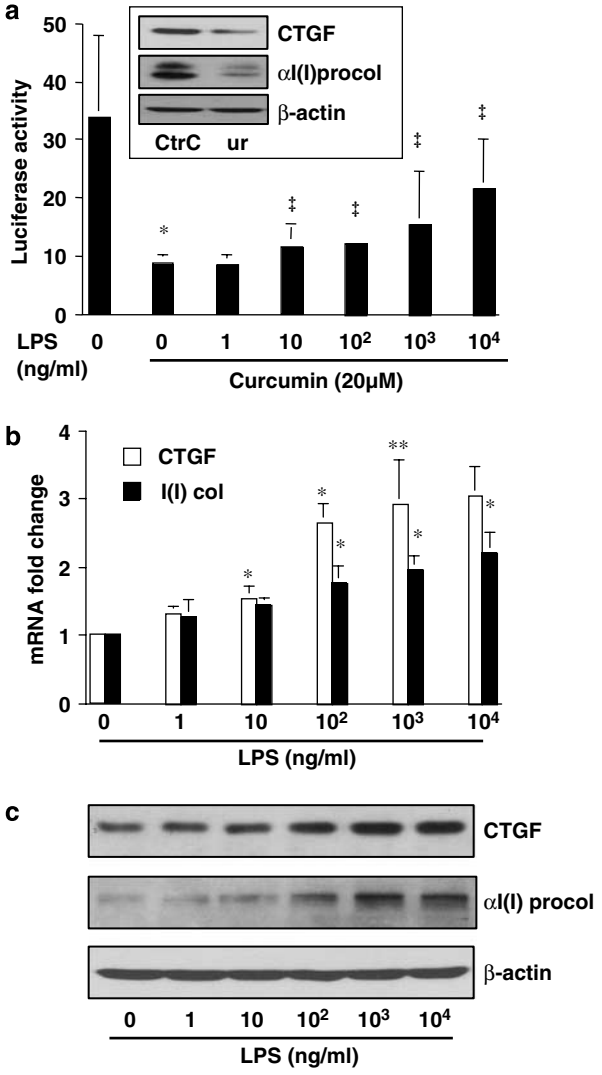

Figure 1 Lipopolysaccharide (LPS) dose-dependently abolishes the inhibitory effect of curcumin on the connective tissue growth factor (CTGF) gene promoter and induces gene expression of CTGF and αI(I) collagen in activated hepatic stellate cell (HSC) *in vitro*. Passaged HSCs were treated with LPS at indicated concentrations for 24 h in the presence and absence of curcumin (20 μM). (**a**) Luciferase assays of cells transfected with the plasmid pCTGF-Luc. Luciferase activities were expressed as relative units after β-galactosidase normalization ($n \geqslant 6$). *$P<0.05$ versus cells with no treatment (the first column on the left). ‡$P<0.05$ versus cells with curcumin only (the second column on the left). The inset demonstrates that curcumin (Cur) reduced the protein abundance of CTGF and αI(I) procollagen (αI(I) procol) analysed by western blotting analyses. (**b**) Real-time PCR analyses of the steady-state mRNA levels of CTGF and αI(I)collagen (αI(I) col). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control for calculating mRNA fold changes. Values are expressed as means ± s.d. ($n=3$). *$P<0.05$, versus the untreated control (the corresponding first column on the left). (**c**) Western blotting analyses of the abundance of CTGF and αI(I)procollagen (αI(I) procol). β-Actin was used as an invariant control for equal loading. Representative blots from three independent experiments are shown.

induce gene expression of CTGF and partially reverse the inhibitory effect of curcumin on the *ctgf* promoter in activated HSC *in vitro*.

NF-κB activity plays a critical role in regulating the promoter activity of CTGF gene in activated HSC in vitro

Further experiments were conducted to directly evaluate the role of NF-κB activity in regulating the promoter activity of CTGF gene in activated HSC *in vitro*. Passaged HSCs were co-transfected with the *ctgf* promoter luciferase reporter plasmid pCTGF-Luc plus a cDNA expression plasmid pCMV-IKK-2 S177E/S181E (pa-IKK2) or pCMV-IKK-2-WT (pIKK2-WT). These two plasmids, respectively, express the constitutively active form of IKK2 or wild-type IKK2, leading to the induction of NF-κB activation. Prior transfection assays demonstrated that pa-IKK2 or pIKK2-WT enhanced NF-κB activity by approximately 20-fold or 1.5-fold, respectively (Yu *et al*., 2006). A total of 3.5 μg of plasmid DNA per well was used for co-transfection of HSC in six-well culture plates. It included 2 μg of pCTGF-Luc, 0.5 μg of pSV-β-gal and 1.0 μg of the cDNA expression plasmid at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were treated with or without curcumin for 24 h. Results from luciferase assays demonstrated that the induction of NF-κB activation by overexpression of constitutively active form of IKK2 (a-IKK2), or wild-type IKK2 (IKK2-WT), dose-dependently eliminated the inhibitory effect of curcumin on the promoter activity of CTGF (Figure 2a). These results also showed that the IKK2-WT was relatively less effective, as expected (Yu *et al*., 2006). These results suggested that the activation of NF-κB might stimulate gene expression of CTGF in passaged HSC.

To verify the role of NF-κB activity in regulating CTGF gene expression, passaged HSCs were similarly co-transfected with pCTGF-Luc plus another cDNA-expressing plasmid pCMV-IκBα-M or pCMV-IκBα-WT. The plasmid pCMV-IκBα-M encodes the dominant-negative form of IκBα (dn-IκBα), leading to the specific blockade of NF-κB activation (Brown *et al*., 1995). The plasmid pCMV-IκBα-WT expresses wild-type IκBα (IκBα-WT), which was used as a control. A total of 4.5 μg of plasmid DNA per well was used for co-transfection of HSC in six-well culture plates. It included 2 μg of pCTGF-Luc, 0.5 μg of pSV-β-gal and 2.0 μg of the cDNA expression plasmid at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After overnight recovery, cells were cultured for an additional 24 h. Luciferase assays demonstrated that forced expression of wild-type IκBα had no significant impact on the *ctgf* promoter activity (Figure 2b). However, the specific inhibition of NF-κB activation by forced expression of dn-IκBα caused a dose-dependent reduction in the *ctgf* promoter activity (Figure 2b), suggesting that the inhibition of NF-κB activity might suppress gene expression of CTGF in passaged HSC. Taken together, our results in Figure 2 demonstrated that the alteration in the activity of NF-κB significantly influenced the *ctgf* promoter activity, suggesting that NF-κB might play a critical role in the regulation of CTGF gene expression in activated HSC *in vitro*.

Activation of PPARγ signalling reduces the activity of NF-κB in activated HSC in vitro

Activation of PPARγ was required for curcumin to inhibit gene expression of CTGF (Zheng and Chen, 2006) and it was therefore likely that curcumin activation of PPARγ signalling might also be required for its inhibition of NF-κB, leading to

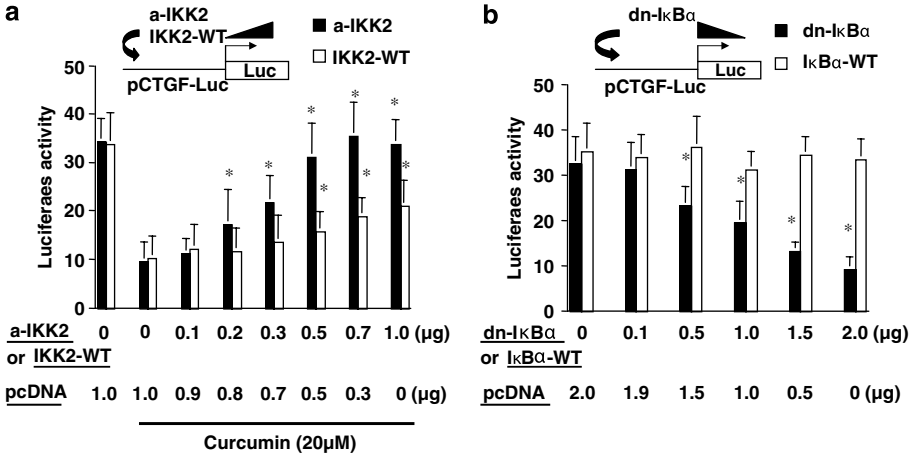

Figure 2 Nuclear factor kappa B (NF-κB) activity plays a critical role in regulating the promoter activity of connective tissue growth factor (CTGF) gene in activated hepatic stellate cell (HSC) *in vitro*. Passaged HSCs were co-transfected with the *ctgf* promoter luciferase reporter plasmid pCTGF-Luc plus a cDNA expression plasmid. A total of 3.5 μg (**a**) or 4.5 μg (**b**) of plasmid DNA per well was used for co-transfection of HSC in six-well culture plates. It included 2 μg of pCTGF-Luc, 0.5 μg of pSV-β-gal and 1.0 μg (**a**) or 2 μg (**b**) of the cDNA expression plasmid at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were treated with or without curcumin for 24 h. Luciferase assays were performed. Luciferase activities were expressed as relative units after β-galactosidase normalization (means ± s.d.; $n \geqslant 6$). (**a**) Luciferase assays of cells co-transfected with pCMV-IKK-2 S177E/S181E (a-IKK2), encoding constitutively active form of IKK2, or with pCMV-IKK-2-WT (IKK2-WT), expressing wild-type IKK2. *$P<0.05$ versus cells transfected with no pa-IKK2 or pIKK2-WT, but treated with curcumin (the second column on the left). (**b**) Luciferase assays of cells co-transfected with pCMV-IκBα-M, encoding dominant-negative form of IκBα (dn-IκBα) or with pCMV-IκBα-WT encoding wild-type IκBα (IκBα-WT). *$P<0.05$ versus cells transfected with no dn-IκBα-M or IκBα-WT (the first column on the left).

the inhibition of *ctgf* expression. To test this assumption, passaged HSCs were transiently transfected with the NF-κB transactivity reporter plasmid pNF-κB-Luc. After recovery, cells were pretreated with or without PD68235, a specific PPARγ antagonist (Camp *et al*., 2001), for 30 min prior to the addition of curcumin (20 μM) for additional 24 h. As shown in Figure 3a by luciferase assays, compared to the untreated control (the first column on the left), curcumin, as expected, significantly reduced luciferase activity (the second column on the left). In contrast, pretreatment of cells with the specific PPARγ antagonist dose-dependently eliminated the inhibitory effect of curcumin (the last two columns on the right). PD68235 itself at 10 or 20 μM showed no significant effect on luciferase activity. These results suggested that the activation of PPARγ might be required for the curcumin inhibition of the NF-κB transactivation activity in passaged HSC. To verify the role of activation of PPARγ in the inhibition of NF-κB activity, passaged HSCs were co-transfected with the plasmid pNF-κB-Luc and the PPARγ cDNA-expressing plasmid pPPARγcDNA at indicated doses. A total of 4.5 μg of plasmid DNA per well was used for the co-transfection of HSC in six-well culture plates. It included 2 μg of pNF-κB-Luc, 0.5 μg of pSV-β-gal and 2 μg of pPPARγcDNA at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were cultured for 24 h. As shown in Figure 3b, forced expression of exogenous PPARγ cDNA dose-dependently reduced luciferase activity, indicating the reduction in the transactivation activity of NF-κB. The inhibitory effect of exogenous PPARγ cDNA was abrogated by treatment of cells with the PPARγ antagonist PD68235 (data not shown). Prior experiments have suggested that 10% of FBS in the medium might contain enough agonists to activate PPARγ in HSC (Miyahara *et al*., 2000; Xu *et al*., 2003; Zheng and Chen, 2004, 2006). To further confirm the inhibitory role of PPARγ signalling, HSCs transfected with pNF-κB-Luc were treated with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a natural PPARγ ligand, at indicated concentrations for 24 h. It was observed that the activation of PPARγ signalling by 15d-PGJ_2 dose-dependently reduced the transactivation activity of NF-κB in HSC, which could be eliminated by pretreatment with PD68235 (the last column on the right) (Figure 3c). PD68235 itself at 20 μM showed no significant effect on luciferase activity (data not shown here). Taken together, these results demonstrated that the activation of PPARγ signalling reduced the transactivation activity of NF-κB in activated HSC *in vitro*. Assay of the cytotoxicity of the drug mixture used here showed that the mixture of the chemicals made no significant difference in the level of LDH in the medium and in the number of Trypan blue-stained HSCs compared with the untreated control. Cell growth rapidly recovered after withdrawal of the chemicals (data not shown). It was, therefore, concluded that the chemicals in this study were not toxic to cultured HSCs.

Activation of PPARγ suppresses gene expression of TLR4 in activated HSC in vitro

TLR4, which acts as an LPS receptor, is involved in the activation of HSC (Paik *et al*., 2003). We therefore evaluated

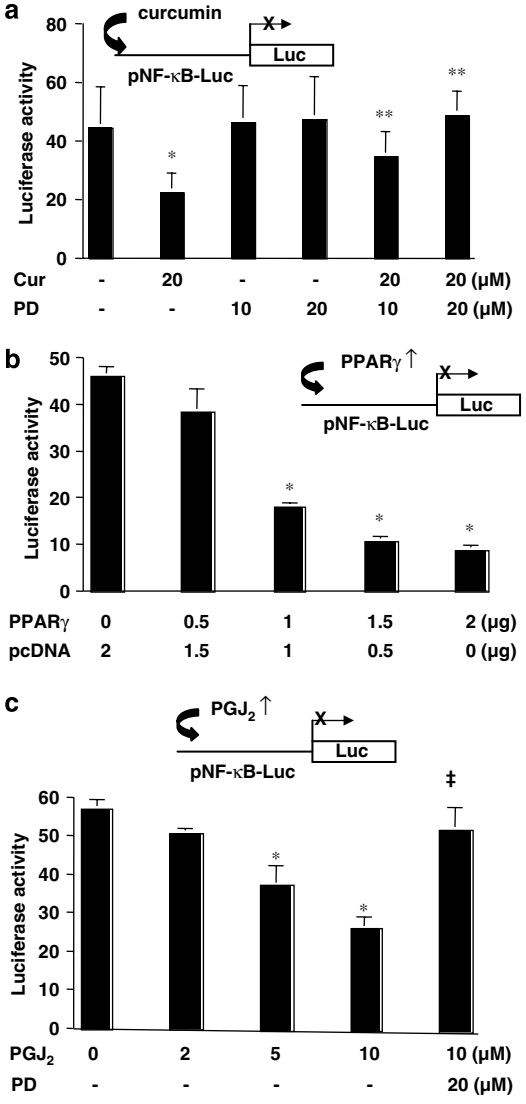

Figure 3 Activation of peroxisome proliferator-activated receptor gamma (PPARγ) reduces nuclear factor kappa B (NF-κB) activity in activated hepatic stellate cell (HSC) *in vitro*. Semiconfluent HSCs were transiently transfected with the plasmid pNF-κB-Luc. After overnight recovery, cells were pretreated with or without the specific PPARγ inhibitor PD68235 (10 or 20 μM) for 30 min prior to the treatment as indicated in the following for additional 24 h. Luciferase assays were performed. Luciferase activities were expressed as relative units after β-galactosidase normalization (means ± s.d.; $n \geqslant 6$). *$P<0.05$ versus cells with no treatment (the first column on the left). **$P<0.05$ versus cells with curcumin only (the second column on the left). The insets denote the pNF-κB-Luc construct in use and the application of a treatment, or a co-transfected plasmid, to the system. (**a**) Cells were treated with curcumin (Cur; 20 μM) with or without PD68235 (PD; 10 or 20 μM). (**b**) Cells were co-transfected with the PPARγ cDNA expression plasmid pPPARγ at indicated doses. A total of 4.5 μg of plasmid DNA per well was transfected to HSC in six-well culture plates. It included 2 μg of pNF-κB-Luc, 0.5 μg of pSV-β-gal and 2 μg of pPPARγ at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After overnight recovery, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS) (10%) for 24 h with no additional treatment. (**c**) Cells were treated with the natural PPARγ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2) at the indicated concentrations with or without PD68235 (20 μM).

the effects of curcumin on gene expression of TLR4 in activated HSC. Semiconfluent passaged HSCs were treated with curcumin at indicated concentrations for 24 h with or without pretreatment with the PPARγ antagonist PD68235 for 30 min. Total RNA and whole-cell extracts were prepared from these cells. As shown in Figure 4 by real-time PCR and western blotting analyses, respectively, curcumin significantly and dose-dependently reduced gene expression of TLR4 in activated HSC *in vitro* (Figures 4a and b). The inhibitory effect was eliminated by the pretreatment with PD68235, suggesting that the activation of PPARγ signalling might play a critical role in the curcumin inhibition of TLR4 gene expression. Further experiments revealed that the activation of PPARγ by 15d-PGJ_2 caused a dose-dependent reduction in the abundance of TLR4 in HSC (Figure 4c). Pretreatment with the PPARγ antagonist dramatically reduced the inhibitory effect caused by 15d-PGJ_2, as well as by curcumin (Figure 4c). These results collectively suggested that the activation of PPARγ by curcumin might result in the suppression of gene expression of TLR4, which could lead to the inhibition of NF-κB activity in activated HSC *in vitro*.

Interruption by curcumin of the ERK signalling pathway suppresses CTGF gene expression in HSC

The ERK signalling pathway plays a key role in the activation of HSC (Pinzani, 2002; Perez de Obanos *et al*., 2006; Zhou *et al*., 2007). We evaluated the effect of curcumin on the ERK signalling pathway and its role in the inhibition of gene expression of CTGF in HSC. Pilot experiments revealed that FBS (10%) rapidly stimulated ERK activity in serum-starved HSC, as demonstrated by the increase in the level of phosphorylated ERK (Figure 5a). The peak of ERK activation is probably within 20 min after FBS stimulation. To evaluate the effect of curcumin on ERK activity, serum-starved HSCs were pretreated with curcumin at indicated concentrations for 30 min prior to stimulation with FBS (10%) for an additional 20 min. Whole-cell extracts were prepared for western blotting analyses. As shown in Figure 5b, the level of phosphorylated ERK was significantly and dose-dependently reduced by curcumin, suggesting that curcumin might inhibit ERK activity in these cells. To determine the role of the curcumin inhibition of ERK activity in the regulation of gene expression of CTGF, semiconfluent HSCs were treated with curcumin at 20 μM or with the specific ERK inhibitor PD98059 at various concentrations for 24 h. Total RNA and whole-cell extracts were prepared from these cells. As shown in Figures 6a and b by real-time PCR and western blotting analyses, respectively, PD 98059, like

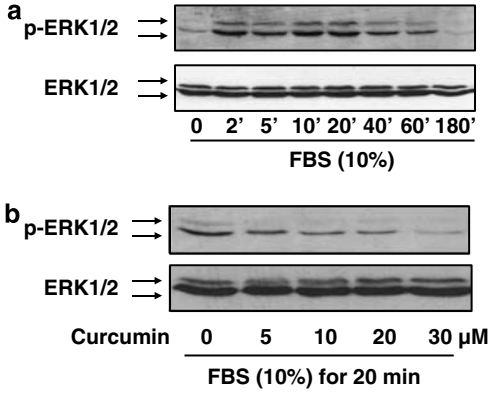

Figure 5 Curcumin inhibits the activation of ERK in activated hepatic stellate cell (HSC) *in vitro*. After serum starvation for 48 h, HSCs were stimulated with fetal bovine serum (FBS) (10%). Whole-cell extracts were prepared for western blotting analyses of the level of phosphorylated ERK1/2 (p-ERK1/2). Total ERK1/2 was used as an internal invariant control for equal loading. Representative blots from three independent experiments are shown. (**a**) Serum-starved cells were stimulated with FBS (10%) for indicated minutes. (**b**) Serum-starved cells were pretreated with curcumin at indicated concentration for 30 min prior to the stimulation with FBS (10%) for additional 20 min.

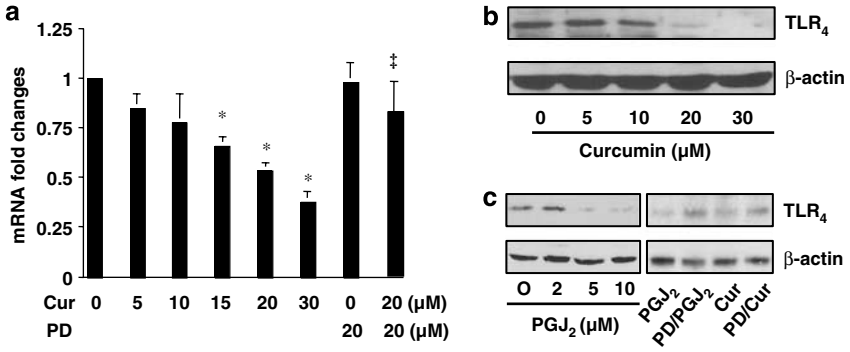

Figure 4 Activation of peroxisome proliferator-activated receptor-gamma (PPARγ) suppresses gene expression of TLR4 in activated hepatic stellate cell (HSC) *in vitro*. Semiconfluent HSCs were pretreated with or without the specific PPARγ inhibitor PD68235 (20 μM) for 30 min prior to the treatment as indicated, for an additional 24 h. Total RNA or whole-cell extracts were prepared from these cells. **(a)** Real-time PCR analyses of the steady-state levels of TLR4 in cells treated with curcumin at indicated concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control for calculating mRNA fold changes. Values are expressed as means ± s.d. ($n = 3$). *$P < 0.05$ versus the untreated control (the first column on the left). **(b)** Western blotting analyses of the abundance of TLR4 in cells treated with curcumin at indicated concentrations. β-Actin was used as an invariant control for equal loading. Representative blots from three independent experiments are shown. (**c**) Western blotting analyses of the abundance of TLR4 in cells treated with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (PGJ_2) at indicated concentrations, or with 15d-PGJ_2 (10 μM) or curcumin (Cur; 20 μM), with or without the pre-exposure to PD68235 (PD; 20 μM). β-Actin was used as an invariant control for equal loading. Representative blots from three independent experiments are shown.

curcumin (the second column, or well), significantly and dose-dependently reduced the steady-state level of mRNA and the protein abundance of CTGF and αI(I) procollagen, indicating that the inhibition of ERK activity resulted in the suppression of gene expression of CTGF and αI(I) collagen in HSC. These results collectively suggested that the curcumin interruption of the ERK signalling pathway might result in the suppression of gene expression of CTGF in HSC.

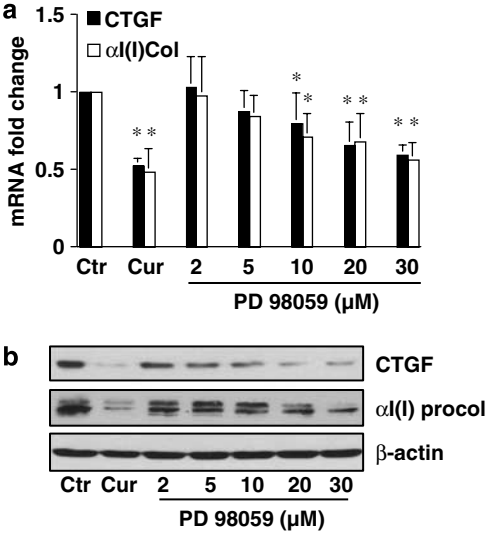

Figure 6 The inhibition of ERK activity by PD98059 suppresses gene expression of connective tissue growth factor (CTGF) and αI(I) collagen in activated hepatic stellate cell (HSC) *in vitro*. Serum-starved cells were pretreated with curcumin (Cur) at 20 μM or with PD98059 at indicated concentrations for 30 min prior to the stimulation with fetal bovine serum (FBS) (10%) for additional 24 h. Total RNA or whole-cell extracts were prepared from these cells. (**a**) Real-time PCR analyses of the steady-state levels of CTGF and αI(I) procollagen (αI(I) col). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control for calculating mRNA fold changes. Values are expressed as means ± s.d. ($n=3$). *$P<0.05$, versus the untreated control (the corresponding first column on the left). (**b**) Western blotting analyses of the abundance of CTGF and αI(I) procollagen (αI(I) procol). β-Actin was used as an invariant control for equal loading. Representative blots from three independent experiments are shown.

To confirm the role of ERK in the regulation of CTGF gene expression, HSCs were co-transfected with pCTGF-Luc plus a cDNA-expressing plasmid of pa-ERK or pdn-ERK. The cDNA expression plasmid pa-ERK, or pdn-ERK, contains a full length of cDNA encoding the constitutively a-ERK or the dominant-negative form of ERK (dn-ERK), respectively (Davis *et al.*, 1996). A total of 3.2 or 4.5 μg of plasmid DNA per well was used for the co-transfection of HSC in six-well culture plates. It included 2 μg of pCTGF-Luc, 0.5 μg of pSV-β-gal and 0.7 μg (Figure 7a) or 2 μg (Figure 7b) of a cDNA expression plasmid at indicated doses plus the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After recovery, cells were serum-starved in DMEM for 24 h prior to the stimulation with FBS (10%) in the presence and absence of curcumin (20 μM) for additional 24 h. As shown in Figure 7a by luciferase assays, compared with the untreated control (the first column on the left), curcumin (the second column on the left), as expected, dramatically reduced luciferase activity. The activation of ERK by forced expression of a-ERK dose-dependently increased luciferase activity and eliminated the inhibitory effect of curcumin on CTGF gene promoter (Figure 7a). On the other hand, the interruption of ERK signalling by expression of dn-ERK in Figure 7b, like curcumin (the last column on the right), dose-dependently reduced luciferase activity, suggesting the suppression of the *ctgf* promoter activity. Taken together, our results demonstrated that the inhibition of ERK activity by curcumin resulted in the suppression of CTGF gene expression in HSC.

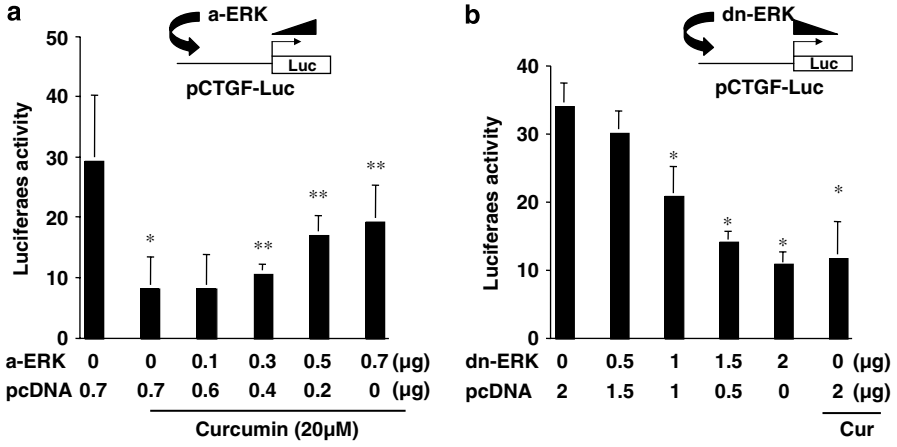

Figure 7 ERK activity plays a role in the regulation of the promoter activity of connective tissue growth factor (CTGF) in activated hepatic stellate cell (HSC) *in vitro*. HSCs were co-transfected with pCTGF-Luc (2 μg per well) plus a cDNA-expressing plasmid of pa-ERK (a-ERK) or pdn-ERK (dn-ERK) at indicated doses. The empty vector pcDNA was used to ensure an equal amount of total DNA in transfection assays. After overnight recovery, cells were serum-starved in Dulbecco's modified Eagle's medium (DMEM) for 24 h prior to the stimulation with fetal bovine serum (FBS) (10%) in the presence and absence of curcumin (20 μM) for additional 24 h. Luciferase assays were performed. Luciferase activities were expressed as relative units after β-galactosidase normalization (means ± s.d.; $n \geqslant 6$). *$P<0.05$ versus cells with no treatment (the first column on the left); **$P<0.05$ versus cells transfected with pcDNA only (the second column on the left). (**a**) Luciferase assays of cells co-transfected with pCTGF-Luc plus pa-ERK. (**b**) Luciferase assays of cells co-transfected with pCTGF-Luc plus pdn-ERK.

Inhibition of ERK activity by curcumin facilitates the reduction in NF-κB activity in HSC

Studies have suggested a cross-talk between the signalling pathways of ERK and NF-κB (Aga *et al*., 2004; Kefaloyianni *et al*., 2006). We postulated that the inhibition of ERK activity by curcumin might facilitate the reduction in NF-κB activity, leading to the inhibition of CTGF gene expression in HSC. To assess this postulate, HSCs were co-transfected with the NF-κB activity reporter plasmid pNF-κB-Luc plus a cDNA-expressing plasmid of pa-ERK or pdn-ERK at indicated doses.

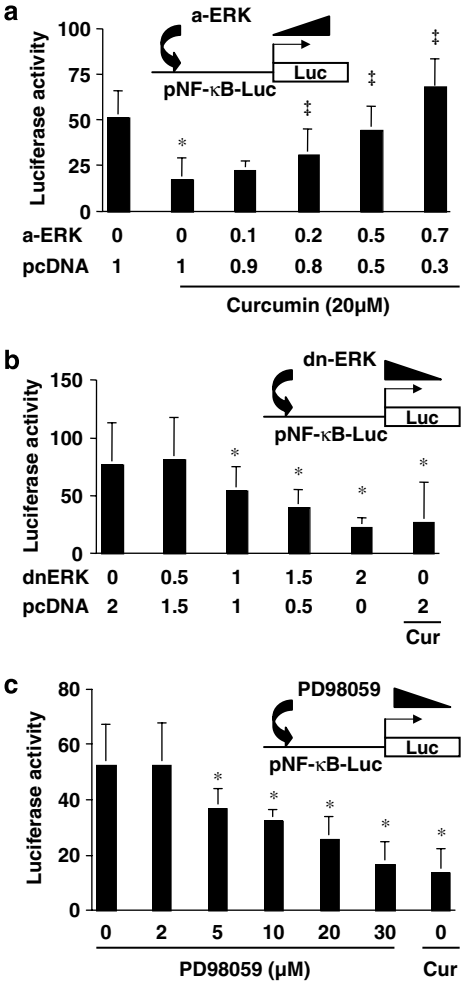

Figure 8 ERK activity affects the transactivation activity of nuclear factor kappa B (NF-κB) in activated hepatic stellate cell (HSC) *in vitro*. HSCs were co-transfected with the plasmid pNF-κB-Luc plus pa-ERK or pdn-ERK at indicated doses. After recovery, cells were serum-starved in Dulbecco's modified Eagle's medium (DMEM) for 24 h prior to the stimulation with fetal bovine serum (FBS) (10%) in the presence and absence of curcumin (20 μM) for additional 24 h. Luciferase assays were performed. Luciferase activities were expressed as relative units after β-galactosidase normalization (means ± s.d.; $n \geqslant 6$). *$P<0.05$ versus cells with no treatment (the first column on the left); ‡$P<0.05$ versus cells transfected with pcDNA only (the second column on the left). (**a**) Luciferase assays of cells co-transfected with pNF-κB-Luc plus pa-ERK. (**b**) Luciferase assays of cells co-transfected with pNF-κB-Luc plus pdn-ERK. (**c**) Luciferase assays of cells transfected with pNF-κB-Luc only, and then treated with the ERK inhibitor PD98059 at indicated concentrations for 24 h.

After recovery, cells were serum-starved in DMEM for 24 h prior to the stimulation with FBS (10%) in the presence and absence of curcumin (20 μM) for additional 24 h. As shown in Figure 8a by luciferase assays, compared with the untreated control (the first column on the left), curcumin (the second column on the left), as expected, dramatically reduced luciferase activity. The activation of ERK by a-ERK dose-dependently eliminated the inhibitory effect of curcumin on NF-κB activity (Figure 8a). On the other hand, the interruption of ERK signalling by dn-ERK, like curcumin (the last column on the right), dose-dependently reduced luciferase activity, suggesting the suppression of NF-κB activity (Figure 8b). To further confirm the effect of ERK activity on NF-κB activity, HSCs transfected with pNF-κB-Luc were treated with the specific ERK inhibitor PD98059 at indicated concentrations for 24 h. Luciferase assays demonstrate that PD98059, like curcumin (the last column on the right), dose-dependently suppressed NF-κB activity (Figure 8c). These results collectively suggested that the inhibition of ERK activity by curcumin might result in the reduction in NF-κB activity, leading to the suppression of CTGF gene expression in HSC.

Discussion

Suppression of HSC activation and prevention of hepatic fibrogenesis attract the attention of researchers from the therapeutic perspective. Many of them have focused their attention on searching for novel agents with inhibitory effects on HSC activation (Friedman, 2004). We recently showed that curcumin inhibited gene expression of CTGF, leading to the reduction in the production of ECM components in activated HSC (Zheng and Chen, 2006). The purpose of this study was to investigate signal transduction pathways involved in the curcumin suppression of CTGF gene expression in activated HSC. To analyse the promoter activity of CTGF, the plasmid pCTGF-Luc, containing a fragment of the CTGF gene promoter (~2000 bp nucleotides), was used in most of this study. In this study, we demonstrated the critical role of NF-κB activity in the regulation of CTGF gene expression in HSC. Curcumin suppressed gene expression of TLR4, leading to the inhibition of NF-κB activity. In addition, our results revealed the effect of ERK activation on NF-κB activity and on the regulation of CTGF gene expression in HSC. Taken together, the present results supported our hypothesis and demonstrated that the inhibition of NF-κB and ERK signalling pathways by curcumin resulted in the suppression of CTGF gene expression in activated HSC.

We are aware that the absorption of dietary curcumin is relatively low and curcumin disappears from rodent tissues relatively rapidly (Perkins *et al*., 2002). Curcumin is unstable *in vivo* and its derivatives might function as the active forms of curcumin *in vivo* (Pan *et al*., 1999). Its effective concentration and its effective metabolites *in vivo* remain largely unknown (Pan *et al*., 1999). The cytotoxicity of curcumin to cultured HSC was evaluated previously (Xu *et al*., 2003). Based on results from LDH release assays, Trypan blue exclusion assays and a rapid recovery of cell proliferation

after withdrawal of curcumin, it was concluded that curcumin up to 100 μM was not toxic to cultured HSC. Curcumin at 20 μM or lower has been chosen for most of our studies in this study, which is much higher than those observed in blood and/or tissues of human and animals (Ammon and Wahl, 1991; Pan *et al*., 1999). However, it must be noted that because the *in vivo* system is multifactorial, directly extrapolating *in vitro* conditions and results, for example, effective concentrations, to the *in vivo* system might be misleading. We recently observed that oral administration of curcumin at 200 mg/kg body weight significantly reduced the level of the transcript and protein of CTGF in the liver, inhibited HSC activation and protected the liver from injury caused by CCl_4 (unpublished data).

We and others have demonstrated previously that curcumin inhibits NF-κB activity in HSC and other cell types (Xu *et al*., 2003; Kamat *et al*., 2007; Shakibaei *et al*., 2007). In the present study, we demonstrated that the inhibition of NF-κB by curcumin played a critical role in the suppression of CTGF gene expression in activated HSC *in vitro*. Our results are consistent with prior observations (Bourgier *et al*., 2005; Hong *et al*., 2006). Two potential NF-κB-binding sites were located within −545 to −535 and −94 to −83 in the promoter of the gene encoding CTGF (Fu *et al*., 2001). Our promoter deletion assays confirmed the necessity of these two regions containing the two NF-κB-binding sites in responding to curcumin (data not shown here). Additional experiments are being conducted in our lab to clarify proteins binding to the sites using site-directed mutagenesis, gel shift assays and chromosomal immunoprecipitation. Other reports also showed that hypoxia increased NF-κB activity, leading to the induction of expression of CTGF in scleroderma skin fibroblasts (Hong *et al*., 2006). Inhibition of Rho kinase reduced the level of CTGF mediated by the inhibition of NF-κB, leading to the reduced expression of type I collagen gene in intestinal smooth muscle cells (Bourgier *et al*., 2005). However, our study has limitations and additional experiments are required.

It remains largely unknown how curcumin suppresses the activity of NF-κB, leading to the suppression of CTGF gene expression. The IκB-α/NF-κB pathway is redox-sensitive and LPS induces NF-κB activation through changes in the redox equilibrium (Haddad and Land, 2002). Inhibition of glutathione biosynthesis by L-buthionine-(*S*,*R*)-sulphoximine blocks the LPS-induced phosphorylation of IκB-α, reduces its degradation and inhibits NF-κB activation (Haddad and Land, 2002). We recently demonstrated that curcumin was a potent antioxidant (Zheng *et al*., 2007). It induced gene expression of glutamate-cysteine ligase, the rate-limiting enzyme in the synthesis of glutathione, in cultured HSC (Zheng *et al*., 2007). Additional experiments are ongoing in our laboratory to evaluate the role of the antioxidant capability of curcumin in the inhibition of NF-κB and in the suppression of CTGF gene expression in HSC.

Activation of HSC is coupled with a dramatic reduction in the level of PPARγ and its activity *in vitro* and *in vivo* (Galli *et al*., 2000; Marra *et al*., 2000; Miyahara *et al*., 2000). Stimulating PPARγ activity by its agonists inhibits HSC proliferation and α1(I) collagen expression *in vitro* and *in vivo* (Miyahara *et al*., 2000; Galli *et al*., 2002). Forced expression of exogenous PPARγ cDNA is sufficient to reverse the morphology of activated HSC to the quiescent phenotype (Hazra *et al*., 2004). We demonstrated previously that curcumin induced gene expression of endogenous PPARγ and stimulated its activity in activated HSC *in vitro* (Xu *et al*., 2003), which was a prerequisite for curcumin to inhibit cell growth and to suppress gene expression of ECM (Xu *et al*., 2003; Zheng and Chen, 2004, 2006). The impact of the curcumin activation of PPARγ on NF-κB activity was studied here. Our results suggested that the activation of PPARγ resulted in the inhibition of NF-κB activity in activated HSC, which might be mediated by the suppression of gene expression of TLR4. TLR4, responsible for the recognition of LPS, has been known to mediate LPS-induced cellular signalling through activation of NF-κB pathway (Eun *et al*., 2006). TLR4 is hardly detectable in quiescent HSC but its expression is highly induced in activated HSC (Paik *et al*., 2003). TLR4 mediates inflammatory signalling by LPS, leading to the activation of NF-κB during hepatic fibrogenesis (Paik *et al*., 2003). Our results are compatible with prior reports. Activation of PPARγ or TLR4 could initiate two antagonistic signalling pathways in intestinal epithelial cells. They may be partially crosslinked (Eun *et al*., 2006), as PPARγ ligand delays LPS-induced IκBα degradation and reduces TLR4 expression. On the other hand, TLR4 negatively regulates PPARγ expression and its anti-inflammatory properties in the development of inflammatory bowel disease (Rousseaux and Desreumaux, 2006). Additional experiments are necessary to elucidate the mechanisms of PPARγ in the regulation of TLR4 gene expression (Dubuquoy *et al*., 2003).

We further evaluated the role of the ERK signalling pathway in the curcumin inhibition of CTGF gene expression. Results in this study demonstrated that the curcumin inhibition of ERK activity resulted in the reduction in NF-κB activity and in the expression of CTGF gene in activated HSC *in vitro*. Our results are consistent with prior studies (Mulsow *et al*., 2005; Shimo *et al*., 2006; Yuan *et al*., 2007). Taurine, a non-essential amino acid, promoted CTGF gene expression in osteoblasts through the activation of the ERK signalling pathway (Yuan *et al*., 2007). Pretreatment of osteoblasts with the ERK inhibitor PD98059 abolished the taurine-induced CTGF production. CTGF is transcriptionally regulated by TGF-β (Fu *et al*., 2001; Blom *et al*., 2002; Leask *et al*., 2003) and this regulation might be Ras/MEK/ERK dependent (Phanish *et al*., 2005). We reported previously that curcumin suppressed CTGF gene expression in activated HSC by interruption of TGF-β signalling (Zheng and Chen, 2006) and there is evidence for cross-talk between the ERK signalling pathway and TGF-β signalling (Mulder, 2000; Wang *et al*., 2005; Huo *et al*., 2007), as well as NF-κB signalling (Kefaloyianni *et al*., 2006). Experiments are ongoing in our laboratory to elucidate mechanisms of ERK and NF-κB signalling pathways in the curcumin interruption of TGF-β signalling, and to further explore mechanisms of cross-talk between them in the regulation of CTGF gene expression in activated HSC.

Based on our observations, a simplified pathway is proposed to describe the possible involvement of signalling pathways in the curcumin inhibition of CTGF gene expression in activated HSC *in vitro*. Curcumin reduces NF-κB

activity by suppressing gene expression of TLR4, which is mediated by activation of PPARγ signalling. In addition, curcumin inhibits ERK activity, which also facilitates the reduction in NF-κB activity. Furthermore, the curcumin inhibition of ERK activity might interfere with other signalling pathways, including TGF-β signalling. These inhibitory effects might collectively reduce the promoter activity of CTGF and suppress its gene expression in activated HSC *in vitro*. Nevertheless, the underlying mechanisms are certainly more complex than it is described here. In addition, our results do not exclude possible involvement of any other signalling pathways and mechanisms in the curcumin inhibition of CTGF gene expression. These results provide novel insights into the mechanisms of curcumin in the inhibition of gene expression of CTGF and ECM components, leading to the inhibition of HSC activation.

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Conflict of interest

The authors state no conflict of interest.

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