

Activation of peroxisome proliferator-activated receptor- γ by curcumin blocks the signaling pathways for PDGF and EGF in hepatic stellate cells

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During hepatic fibrogenesis, reduction in the abundance of peroxisome proliferator-activated receptor- γ (PPAR γ) is accompanied by activation of mitogenic signaling for platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) in hepatic stellate cells (HSCs), the major effector cells. We previously reported that curcumin, the yellow pigment in curry, interrupted PDGF and EGF signaling, stimulated PPAR γ gene expression, and enhanced its activity, leading to inhibition of cell proliferation of activated HSC *in vitro* and *in vivo*. The aim of this study was to elucidate the underlying mechanisms. We hypothesized that the enhancement of PPAR γ activity by curcumin might result in the interruption of PDGF and EGF signaling. Our experiments demonstrated that curcumin, with different treatment strategies, showed different efficiencies in the inhibition of PDGF- or EGF-stimulated HSC proliferation. Further experiments observed that curcumin dose dependently reduced gene expression of PDGF and EGF receptors (ie, PDGF- β R and EGFR), which required PPAR γ activation. The activation of PPAR γ by its agonist suppressed *pdgf- β r* and *egfr* expression in HSC. In addition, curcumin reduced the phosphorylation levels of PDGF- β R and EGFR, as well as their downstream signaling cascades, including ERK1/2 and JNK1/2. Moreover, activation of PPAR γ induced gene expression of glutamate-cysteine ligase, the rate-limiting enzyme in *de novo* synthesis of the major intracellular antioxidant, glutathione. *De novo* synthesis of glutathione was required for curcumin to suppress *pdgf- β r* and *egfr* expression in activated HSCs. Our results collectively demonstrated that enhancement of PPAR γ activity by curcumin interrupted PDGF and EGF signaling in activated HSCs by reducing the phosphorylation levels of PDGF- β R and EGFR, and by suppressing the receptor gene expression. These results provide novel insights into the mechanisms of curcumin in the inhibition of HSC activation and the suppression of hepatic fibrogenesis.

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Hepatic stellate cells (HSCs) are recognized as the major effector cells in the development of hepatic fibrosis.^{1,2} In response to liver injury caused by virus, chemicals, autoimmune reactions, or metabolic disorders, HSCs undergo obvious phenotypic alterations from a state of quiescence to activation.^{1,2} HSC activation is characterized by enhanced cell proliferation, over-production of extracellular matrix (ECM), and *de novo* synthesis of α -smooth muscle actin. This process is coupled with the up-expression of receptors for platelet-derived growth factor (PDGF)³ and epidermal growth factor (EGF),^{4–6} as well as the dramatic down-expression of peroxisome proliferator-activated receptor- γ (PPAR γ).^{7–9} The alteration in PPAR γ activity plays an important role in HSC

activation.^{10,11} HSC proliferation is, in a receptor-dependent manner, triggered by autocrine/paracrine activation of the signaling for mitogenic growth factors, including PDGF and EGF.^{4,12} Both PDGF- β R and EGF receptor (EGFR) contain intrinsic tyrosine kinase activity. Upon binding to their ligands, the receptors become phosphorylated at the tyrosine residues, leading to activation of their downstream cascades, including the extracellular signal-regulated kinase (ERK) and the Jun N-terminal kinase (JNK).^{13,14}

In the last decade, advances in the understanding of genes promoting HSC activation have been impressive. There are, however, few breakthroughs in the therapeutic intervention of hepatic fibrogenesis. Therefore, research identifying

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antifibrogenic agents that are innocuous is of high priority and is urgently needed. Most evolving antifibrogenic therapies will be aimed at inhibiting HSC activation. The antioxidant curcumin is the yellow pigment in the curry from turmeric. It has received attention as a promising dietary supplement for the treatment of hepatic fibrogenesis.^{11,15–22}

We previously reported that curcumin interrupted PDGF and EGF signaling, stimulated gene expression of PPAR γ , and enhanced its activity, leading to inhibition of cell proliferation of activated HSCs *in vitro*.^{11,22} The aim of this study was to elucidate the underlying mechanisms. We hypothesized that enhancement of PPAR γ activity by curcumin might result in interruption of the signaling pathways for PDGF and EGF. Results in this report support our hypothesis and provide novel insights into the mechanisms of curcumin in the inhibition of HSC activation and the suppression of hepatic fibrogenesis.

MATERIALS AND METHODS

Materials and Chemicals

Curcumin (purity > 94%) was purchased from Sigma (St Louis, MO, USA) and dissolved in 100% ethanol. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (PGJ₂), a natural PPAR γ agonist, is the product of Cayman Chemical Company (Ann Arbor, MI, USA). PD98059 (an ERK inhibitor) and SP600125 (a JNK inhibitor) were, respectively, purchased from Biomol Research Labs. Inc (Plymouth Meeting, PA, USA) and Sigma, both of which were dissolved in DMSO. PD68235, a specific PPAR γ antagonist, was generously provided by Pfizer (Ann Arbor, MI, USA). L-Buthionine-sulfoximine (BSO) and N-acetyl-L-cysteine (NAC) were obtained from Sigma. Recombinant PDGF-BB and EGF were purchased from Cell Sciences (Canton, MA, USA).

Isolation and Culture of HSCs

HSCs were isolated from male Spague–Dawley rats (200–250 g) by the pronase–collagenase perfusion *in situ* prior to density gradient centrifugation on an Opti-Prep™ gradient, as previously described.²³ Primary cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). The passaged cells were grown in DMEM with 10% of FBS. Semi-confluent HSCs with 4–8 passages were used for the experiments. In some of the experiments, cells were serum-starved for 24 h in DMEM with 0.5% of FBS before treatment.

Determination of Cell Proliferation

HSCs were serum-starved in DMEM with 0.5% of FBS for 24 h prior to addition of PDGF or EGF at the indicated doses, in the presence or absence of curcumin (20 μ M), for an additional 24 h. In another group, HSCs were pretreated with curcumin (20 μ M) for 24 h in DMEM with 0.5% of FBS prior to addition of PDGF, or EGF, at the indicated doses for an additional 24 h. Cell proliferation was determined either by counting cell numbers using a computer-equipped cell counter (Coulter Corporation, Miami, FL, USA), or using

the CellTiter 96[®] aqueous non-radioactive cell proliferation assay kit (ie, MTS assays) purchased from Promega, according to the manufacturer's instructions. MTS assay is a colorimetric method for determining the number of viable cells. Each treatment was performed in triplicates and was repeated for at least three times.

Western Blotting Analyses

Whole-cell extracts were prepared as described.²³ Proteins were separated by SDS-PAGE with 10% of resolving gel and transferred to a PVDF membrane. The target proteins were respectively recognized by primary antibodies against phosphorylated types of PDGF- β R, EGFR, ERK1/2, or JNK1/2 and the corresponding non-phosphorylated proteins, and subsequently detected by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized with a chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). In western blotting analyses, the densities of bands were normalized with the internal invariable control, β -actin.

RNA Isolation and Real-time Polymerase Chain Reaction

Total RNA was extracted using TRI reagent[®] (Sigma), following the protocol provided by the manufacturer. Real-time polymerase chain reaction (PCR) was carried out as previously described.¹¹ mRNA fold changes of target genes relative to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control were calculated as suggested by Schmittgen *et al.*²⁴ The following primers were used for real-time PCR: PDGF- β R: (F) 5'-CTGCCACAGCATGATGAGGATTGA-3', (R) 5'-GCCAGGATGGCTGAGATCACCAC; EGFR: (F) 5'-GTGACCGTTTGGGAGTTGATGA-3', (R) 5'-GGCTGAGGGAGGCGT TCTC-3'; GCLc: (F) 5'-TGTTGTGATGAGCCCAAGGAC-3', (R) 5'-AGTTGGCTCGCATCATAGTTG-3'; GCLm: (F) 5'-CTGCTAAACTGTTTCATTGTAG-3', (R) 5'-CTATGGGTTTTACCTGTG-3'; and GAPDH: (F) 5'-GGCAAATTC AACGGCACAGT-3', (R) 5'-AGATGGTGTATGGGCTTCCC-3'.

Plasmids and Transient Transfection Assays

The *pdgf- β r* promoter report plasmid, pPDGF- β R-Luc, was a gift from Dr Keiko Funa (Ludwig Institute for Cancer Research, Uppsala, Sweden). It contained a fragment (1366 bp) of PDGF- β R gene promoter subcloned into the luciferase expression vector pGL2 (basic).²⁵ The *egfr* promoter luciferase reporter plasmid contained a fragment (1109 bp) of *egfr* promoter in a luciferase reporter plasmid. It was a gift from Dr Alfred C Johnson (Laboratory of Molecular Biology, NCI/NIH, Bethesda, MD, USA).²⁶ The cDNA-expressing plasmid, pPPAR γ cDNA, contained a full fragment of cDNA encoding human PPAR γ (a gift from Dr Reed Graves, Department of Medicine, University of Chicago, Chicago, IL, USA). The plasmid pdn-ERK, or pdn-JNK, contained a full length of cDNA fragment encoding the dominant-negative form of ERK or JNK, respectively. Both of them were previously described and used.²⁷ The plasmid

pa-ERK, or pa-JNK, contained a full length of cDNA fragment encoding the constitutively active form of ERK, or JNK, respectively.^{27,28} Transient transfection assays were performed using Lipofectamine[®] (Life Technologies) following the protocol provided by the manufacturer. A total of 3.5~4.5 μ g of DNA were added to semi-confluent cells in each well of six-well plastic plates. Transfection efficiency was controlled by co-transfection of the β -galactosidase reporter plasmid, pSV- β -gal (0.5 μ g/well) (Promega). β -Galactosidase activity was measured using an assay kit from Promega Corp. Luciferase activities were expressed as relative unit after normalization with β -galactosidase activity. Results were combined from multiple independent experiments ($n \geq 6$) and were presented as means \pm s.d.

Statistical Analysis

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

RESULTS

Curcumin, with Different Treatment Strategies, showed Different Efficiencies in the Inhibition of PDGF- or EGF-Stimulated HSC Proliferation

PDGF and EGF have been identified as the most potent stimuli for proliferation of HSCs.^{4,12} To evaluate the effect of curcumin on the PDGF-, or EGF-stimulated cell proliferation, cultured HSCs were divided into three groups. In the first two groups, HSCs were serum-starved in DMEM with 0.5% of FBS for 24 h prior to addition of PDGF or EGF at the

indicated doses in the simultaneous presence or absence of curcumin (20 μ M) for an additional 24 h. In the third group, HSCs were pretreated with curcumin (20 μ M) for 24 h in DMEM with 0.5% of FBS prior to addition of PDGF, or EGF, at the indicated doses for an additional 24 h. Cell proliferation was determined following these steps. As shown in Figure 1, in MTS assays exogenous PDGF or EGF caused, as expected, a dose-dependent increase in cell density (the left first columns in each group). The stimulatory effects of PDGF and EGF were diminished by curcumin (the middle and right columns in each group). However, the extent of diminution was obviously different when different strategies were used. Compared with simultaneous treatment with curcumin (the middle black columns), pretreatment with curcumin (the right blank columns) potentiated its role in the suppression of cell proliferation. For instance, compared with treatment with only PDGF at 15 or 20 ng/ml, simultaneous treatment with curcumin (20 μ M) inhibited PDGF-stimulated cell proliferation by 10 or 19%, respectively (Figure 1a). However, pretreatment with curcumin (20 μ M) potentiated the inhibitory effect on PDGF-stimulated cell proliferation by 32 or 43%, respectively (Figure 1a). Similar results were observed in EGF-stimulated cell proliferation when different treatment strategies with curcumin were used (Figure 1b). It bears notice that because experiments were performed in serum-depleted medium, even stimulated with PDGF or EGF, the rate of cell proliferation was relatively low, compared with that of cells grown in DMEM with 10% of FBS. Similar results were obtained using an alternative approach, that is, counting cell numbers using a computer-equipped cell counter (data not shown here). Taken together, these results demonstrated that curcumin, with different

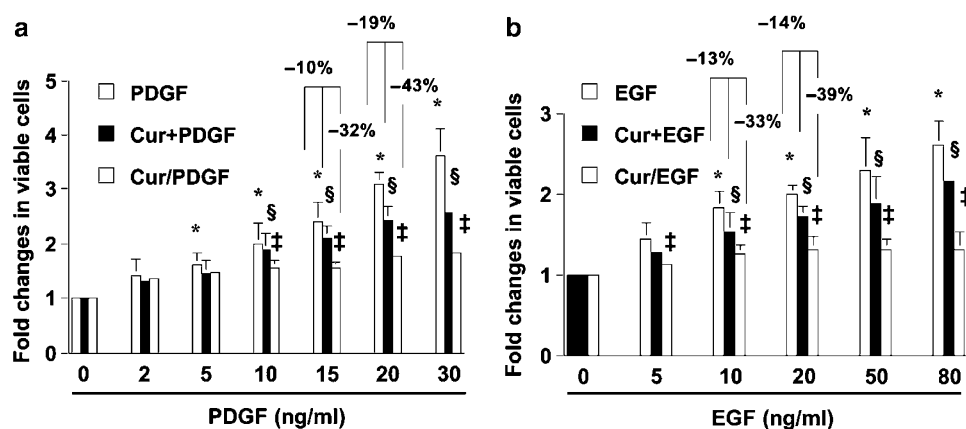


Figure 1 Curcumin, with different treatment strategies, showed different efficiencies in the inhibition of PDGF- or EGF-stimulated HSC proliferation. Cultured HSCs were divided into three groups. In the first two groups, HSCs were serum-starved in DMEM with 0.5% of FBS for 24 h prior to addition of PDGF (a) or EGF (b) at the indicated doses in the simultaneous presence (Cur+PDGF, or Cur+EGF) or absence (PDGF or EGF) of curcumin (20 μ M) for an additional 24 h. In the third group, HSCs were pretreated with curcumin (20 μ M) for 24 h in DMEM with 0.5% of FBS prior to the addition of PDGF (a) or EGF (b) at the indicated doses for an additional 24 h (Cur/PDGF, or Cur/EGF). Cell proliferation was determined by colorimetric MTS assays. Results were expressed as fold changes in the number of viable cells, compared with the untreated control (the first columns on the left). Values were expressed as means \pm s.d. ($n = 3$). * $P < 0.05$ vs the untreated control (the first column on the left). $\ddagger P < 0.05$ vs cells treated with PDGF or EGF only (the left first column in the same group). $\S P < 0.05$ vs cells simultaneously treated with curcumin plus PDGF or EGF (Cur+PDGF, or Cur+EGF) (the middle column in the same group).

treatment strategies, showed different efficiencies in the inhibition of PDGF- or EGF-stimulated HSC proliferation. The intriguing observations urged us to investigate the underlying mechanisms.

Curcumin Inhibited Gene Expression of PDGF β R and EGFR in Activated HSCs *In Vitro*, which Might Require Activation of PPAR γ

Because PDGF and EGF signaling is receptor-dependent, we proposed that the different inhibitory efficacy of curcumin on cell proliferation might result, at least partially, from suppression of gene expression of their receptors in the case of pretreatment. The effect of curcumin on gene expression of PDGF- β R and EGFR in passaged HSCs was, therefore, evaluated. Pre-confluent HSCs in six-well culture plates were transiently transfected with the *pdgf- β r* promoter luciferase reporter plasmid, pPDGF- β R-Luc, or the *egfr* promoter luciferase reporter plasmid, pEGFR-Luc. After recovery, cells were treated with curcumin at various concentrations as indicated for 24 h. As shown in Figure 2a, in luciferase assays curcumin caused a dose-dependent reduction in luciferase activity, suggesting that curcumin might reduce the promoter activity of PDGF- β R and EGFR genes in activated HSCs *in vitro*. To confirm the result, passaged HSCs were treated with curcumin at various concentrations as indicated for 24 h. Real-time PCR (Figure 2b) and Western blotting analyses (Figure 2c) demonstrated that curcumin significantly reduced the mRNA levels and the protein abundance of PDGF- β R and EGFR in a dose-dependent manner. These results demonstrated that curcumin suppressed *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*.

We previously reported that curcumin induced gene expression of PPAR γ and enhanced its activity in cultured HSCs,¹¹ which was a prerequisite for curcumin to inhibit HSC proliferation and to suppress gene expression of ECM components.^{11,19} It was, therefore, plausible to further postulate that inhibition of *pdgf- β r* and *egfr* expression by curcumin might be mediated by activation of PPAR γ . To test the postulation, cultured HSCs were pretreated with or without PD68235 (20 μ M), a specific PPAR γ antagonist, for 1 h prior to addition of curcumin (20 μ M) for an additional 24 h. Gene expression of PDGF β R and EGFR was assessed by real-time PCR (Figure 2b) and Western blotting analyses (Figure 2c). It was revealed that pre-exposure of HSCs to PD68235 obviously diminished the inhibitory effect of curcumin on *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*, suggesting that activation of PPAR γ might be necessary for curcumin to inhibit *pdgf- β r* and *egfr* expression.

Activation of PPAR γ Suppressed *pdgf- β r* and *egfr* Expression in Activated HSCs *In Vitro*

Additional experiments were performed to verify the role of PPAR γ in curcumin suppression of *pdgf- β r* and *egfr* expression. HSCs in six-well culture plates were co-transfected with a total of 4.5 μ g of a DNA mixture per well, including 2 μ g

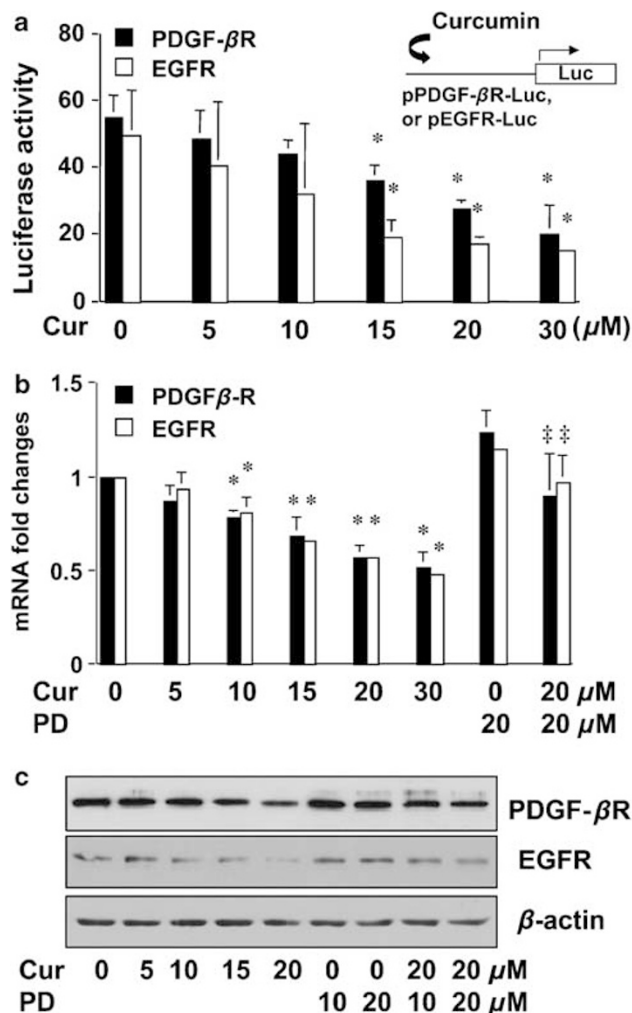


Figure 2 Curcumin dose dependently suppressed gene expression of PDGF- β R and EGFR in cultured HSCs, which might require activation of PPAR γ . Cultured HSCs in DMEM with 10% of FBS were pretreated with or without the PD68235 (20 μ M), a specific PPAR γ antagonist, for 1 h prior to the addition of curcumin at the indicated concentrations for an additional 24 h. (a) Luciferase assays of cells transfected with pPDGF- β R-Luc, or pEGFR-Luc. Luciferase activities were expressed as relative units after β -galactosidase normalization ($n = 6$). * $P < 0.05$ vs the untreated control (the corresponding first column on the left). The floating schema denoted the pPDGF- β R-Luc, or pEGFR-Luc, luciferase reporter construct in use and the application of curcumin to the system; (b) real-time PCR analyses of the mRNA levels of PDGF- β R or EGFR. GAPDH was used as an invariant control for calculating fold changes of target mRNA ($n = 3$). * $P < 0.05$ vs the untreated control (the corresponding first column on the left); [‡] $P < 0.05$ vs cells treated with curcumin at 20 μ M; (c) Western blotting analyses of the protein abundance of PDGF- β R or EGFR. β -Actin was used as an invariant control for equal loading. Representative result is shown from three independent experiments.

of pPDGF- β R-Luc, or pEGFR-Luc, 0.5 μ g of pSV- β -gal, and 2 μ g of the PPAR γ cDNA expressing plasmid pPPAR γ cDNA at various doses plus the empty vector pcDNA. The latter was used to ensure equal amount of total DNA in transfection assays. After recovery, cells were treated with or without curcumin (20 μ M) in media containing FBS (10%) for 24 h. Luciferase activity assays in Figure 3 indicate that forced

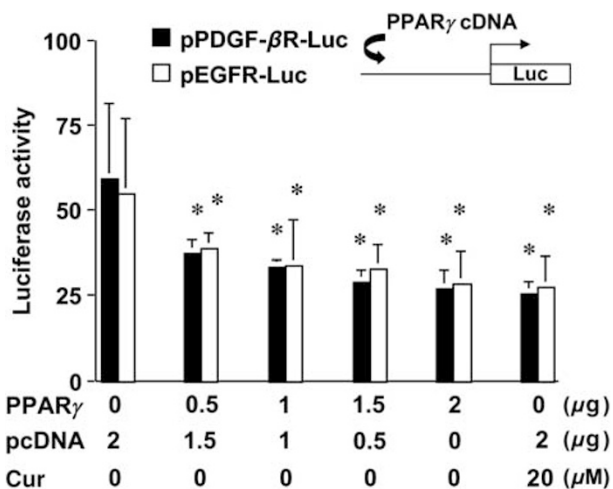


Figure 3 Forced expression of PPAR γ reduced the promoter activity of PDGF- β R and EGFR genes in passaged HSCs. HSCs in six-well culture plates in DMEM with 10% of FBS were co-transfected with a total of 4.5 μ g of a DNA mixture per well, including 2 μ g of pPDGF- β R-Luc or pEGFR-Luc, 0.5 μ g of pSV- β -gal and 2 μ g of pPPAR γ cDNA at various doses plus the empty vector pcDNA. The latter was used to ensure equal amount of total DNA in transfection assays. After recovery, cells were treated with or without curcumin (20 μ M) in media containing FBS (10%) for 24 h. Luciferase activities were expressed as relative units after β -galactosidase normalization ($n = 6$). * $P < 0.05$ vs cells with no treatment (the first column on the left). The floating schema denoted the pPDGF- β R-Luc, or pEGFR-Luc, luciferase reporter construct in use and the application of the cDNA expressing plasmid pPPAR γ cDNA to the system.

expression of exogenous PPAR γ cDNA dose dependently reduced luciferase activity, indicating reduction in the promoter activity of PDGF- β R and EGFR genes in cultured HSCs. The inhibitory effect of exogenous PPAR γ cDNA was abrogated by treatment of cells with the PPAR γ antagonist, PD68235 (data not shown here). Prior experiments have suggested that 10% FBS in the medium might contain enough agonists to activate PPAR γ in HSCs.^{9,11,19,20}

Additional experiments further confirmed that activation of endogenous PPAR γ by its natural agonist, PGJ₂, inhibited *pdgf- β r* and *egfr* promoter activity (Figure 4a), and reduced the mRNA level (Figure 4b) and protein abundance (Figure 4c) of PDGF- β R and EGFR in cultured HSCs. These results demonstrated that activation of PPAR γ suppressed *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*. Taken together, our results implicated that inhibition of *pdgf- β r* and *egfr* expression by curcumin might be mediated by activation of PPAR γ in activated HSCs *in vitro*.

Curcumin Reduced the Phosphorylation Levels of PDGF- β R, EGFR, and of their Downstream Inter-Mediators in HSCs

In addition to suppression of *pdgf- β r* and *egfr* expression, curcumin might have additional mechanisms to interrupt the signaling pathways for PDGF and EGF in HSCs. Additional experiments were conducted to evaluate the effects of curcumin on the tyrosine phosphorylation of PDGF- β R, EGFR,

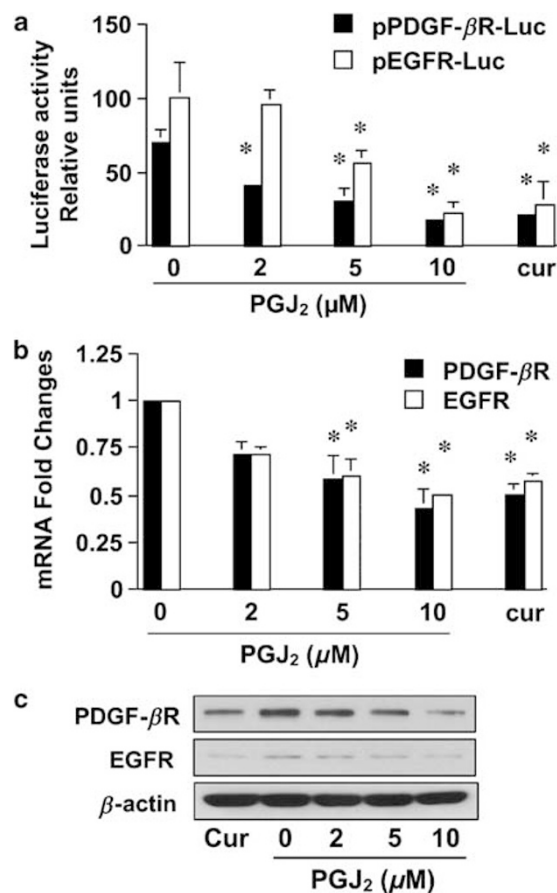


Figure 4 The activation of PPAR γ by PGJ₂ suppressed *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*. Cultured HSCs in DMEM with 10% of FBS were treated with curcumin (20 μ M) or PGJ₂ at the indicated concentrations for 24 h. (a) Luciferase assays of cells transfected with pPDGF- β R-Luc, or pEGFR-Luc. Luciferase activities were expressed as relative units after β -galactosidase normalization ($n = 6$). * $P < 0.05$ vs the untreated control (the corresponding 1st column on the left); (b) real-time PCR analyses of the mRNA levels of PDGF- β R or EGFR. GAPDH was used as an invariant control for calculating fold changes of target mRNA ($n = 3$). * $P < 0.05$ vs the un-treated control (the corresponding 1st column on the left); (c) Western blotting analyses of the abundance of PDGF- β R or EGFR. β -Actin was used as an invariant control for equal loading. Representative result is shown from three independent experiments.

and their downstream cascades. Passaged HSCs were serum-starved in serum-depleted DMEM with 0.5% of FBS for 24 h. Cells were stimulated with FBS (10%) for various minutes, as indicated, with or without pretreatment with curcumin (20 μ M) for 1 h. The phosphorylation levels of the target proteins were evaluated by Western blotting analyses. It is shown in Figure 5 that FBS rapidly stimulated tyrosine phosphorylation of PDGF- β R and EGFR (well nos. 1, 3, 5, 7, and 9), leading to enhanced phosphorylation of the downstream inter-mediators ERK1/2 and JNK1/2, which reached their peaks within 10~20 min (well nos. 3 and 5). Pretreatment of cells with curcumin apparently reduced the phosphorylation levels of PDGF- β R, EGFR, ERK1/2, and JNK1/2 (well nos. 2, 4, 6, 8, and 10), compared with their

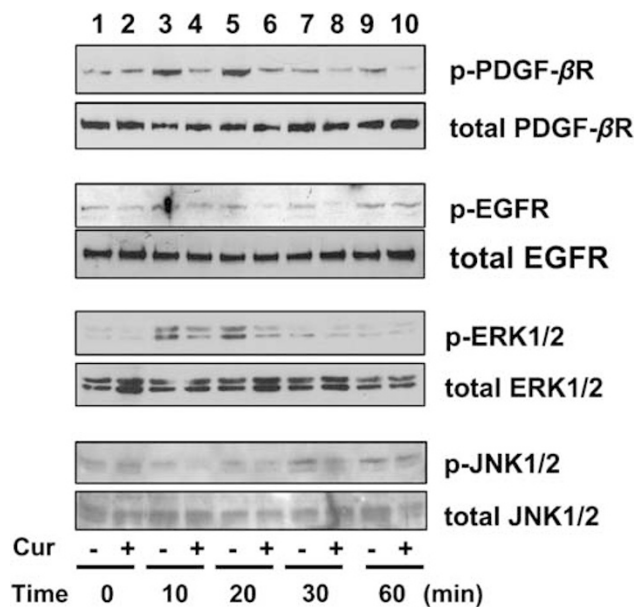


Figure 5 Curcumin reduced the phosphorylation levels of PDGF- β R, EGFR, and downstream inter-mediators ERK and JNK in activated HSC *in vitro*. Serum-starved HSCs in DMEM with 10% of FBS were pretreated with or without curcumin at 20 μ M for 30 min prior to stimulation with FBS (10%) for different lengths of time. Total protein extracts were prepared for detecting the levels of phosphorylated PDGF- β R, EGFR, ERK, and JNK by Western blotting analysis. Total PDGF- β R, EGFR, ERK, and JNK were used as the corresponding internal controls. Representative result is shown from three independent experiments.

corresponding counterparts (well nos. 1, 3, 5, 7, and 9). These results demonstrated that in addition to suppression of *pdgf- β r* and *egfr* expression, curcumin interrupted the signaling pathways for PDGF and EGF in HSCs by reducing the phosphorylation levels of PDGF- β R, EGFR, and their downstream inter-mediators.

Inhibition of ERK or JNK Activity Resulted in Suppression of *pdgf- β r* and *egfr* Expression in Activated HSCs *In Vitro*

We assumed that inhibition of ERK and JNK activity by curcumin could result in suppression of *pdgf- β r* and *egfr* expression in activated HSCs. Experiments were conducted to test the assumption. First experiments were performed to determine whether alteration of ERK or JNK activity could reduce the promoter activity of PDGF- β R and EGFR genes in activated HSCs. HSCs were co-transfected with pPDGF- β R-Luc, or pEGFR-Luc, plus a cDNA expressing plasmid of pa-ERK, pa-JNK, pdn-ERK, or pdn-JNK. The cDNA expressing plasmid pa-ERK, or pa-JNK, contained a fragment of cDNA encoding the constitutively active form of ERK (a-ERK), or JNK (a-JNK), respectively.^{27,28} Similarly, the cDNA expressing plasmid pdn-ERK, or pdn-JNK, contained a fragment of cDNA encoding the dominant-negative form of ERK (dn-ERK) or JNK (dn-JNK), respectively.^{27,28} A total of 3.2 μ g, or 4.5 μ g, of plasmid DNA was used for

co-transfection of HSCs in each well of six-well culture plates. It included 2 μ g of pPDGF- β R-Luc, or pEGFR-Luc, 0.5 μ g of pSV-gal, and 0.7 μ g of pa-ERK or pa-JNK, or 2 μ g of pdn-ERK or pdn-JNK, plus the empty vector, pcDNA. The latter was used to ensure the equal amount of total DNA in co-transfection assays. After overnight recovery, cells were serum-starved in DMEM for 24 h prior to stimulation with FBS (10%) in the presence or absence of curcumin (20 μ M) for an additional 24 h. The effects of ERK or JNK activity on *pdgf- β r* and *egfr* promoter activity were evaluated by luciferase assays. As shown in Figure 6a and c, compared with the untreated control (the corresponding first column on the left), activation of ERK, or JNK, by forced expression of pa-ERK, or pa-JNK, markedly increased luciferase activity (the second columns on the left). Curcumin significantly reduced, as expected, luciferase activity (the third columns on the left in Figure 6a and c). pa-ERK and pa-JNK dose dependently abolished the inhibitory effect of curcumin on luciferase activity (Figure 6a and c). In great contrast, inhibition of ERK or JNK by pdn-ERK or pdn-JNK dose dependently reduced luciferase activity (Figure 6b and d). These results collectively suggested that alteration of ERK or JNK activity regulated the promoter activity of PDGF- β R and EGFR genes in activated HSCs *in vitro*.

Further experiments confirmed that inhibition of ERK, or JNK, by the ERK inhibitor, PD98059, or the JNK inhibitor, SP600125, mimicked the inhibitory effects of curcumin and reduced promoter activity (Figure 7a and b), mRNA levels (Figure 7c and d), and protein abundance (Figure 7e and f) of PDGF- β R and EGFR in cultured HSCs, as demonstrated by co-transfection assays, real-time PCR, and western blotting analyses, respectively. Taken together, these results supported the assumption that inhibition of ERK and JNK activity by curcumin could result in suppression of *pdgf- β r* and *egfr* expression in activated HSCs.

De Novo Synthesis of GSH Played a Critical Role in Curcumin Inhibition of *pdgf- β r* and *egfr* Expression in Activated HSCs

We have previously demonstrated that curcumin dose, and time dependently attenuates oxidative stress in passaged HSCs, as demonstrated by scavenging of reactive oxygen species and reduction of lipid peroxidation.²¹ The anti-oxidant property of curcumin mainly results from increasing in the level of cellular glutathione (GSH) by induction of the activity and gene expression of glutamate-cysteine ligase (GCL), a rate-limiting enzyme in the synthesis of GSH, in activated HSCs *in vitro*. *De novo* synthesis of GSH is a prerequisite for curcumin to inhibit HSC activation.²¹ It was, therefore, plausible to investigate the role of the enhanced level of cellular GSH in the curcumin inhibition of *pdgf- β r* and *egfr* expression in activated HSCs. Passaged HSCs were treated with curcumin (20 μ M), or NAC (5 mM), a precursor of GSH by supplying cysteine,²⁹ with or without the pre-exposure to BSO (0.25 mM), a specific inhibitor of GCL to

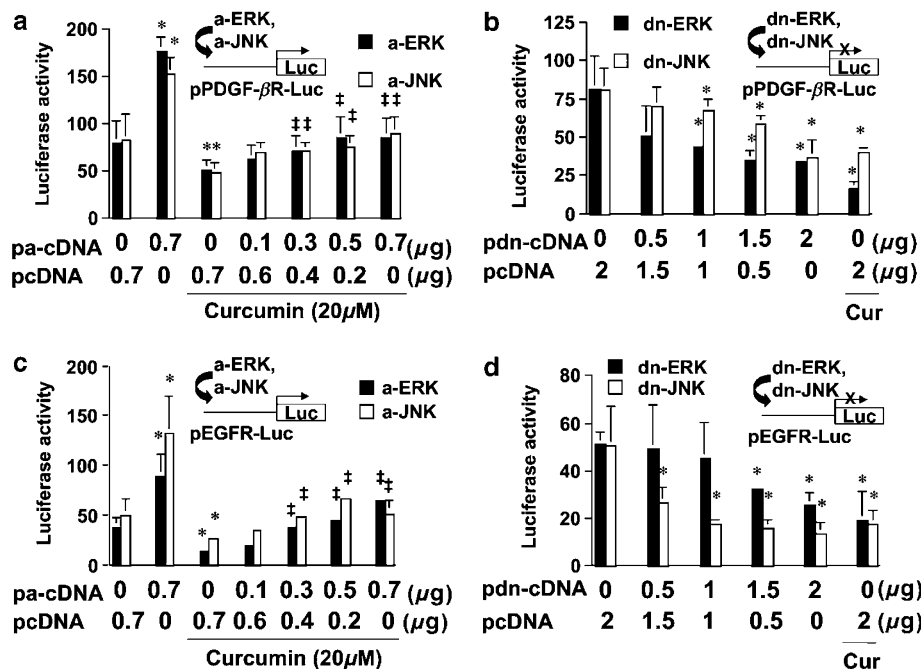


Figure 6 The alteration in ERK or JNK activity changed the promoter activity of PDGF- β R and EGFR genes in activated HSCs *in vitro*. HSCs in six-well plates in DMEM with 10% of FBS were co-transfected with a fixed amount of a DNA mixture per well. It included 2 μ g of pPDGF- β R-Luc (a and b), or pEGFR-Luc (c and d), 0.5 μ g of pSV- β -gal and a plasmid expressing the active form of pa-ERK, or pa-JNK (a and c), or a plasmid expressing the dominant-negative form of pdn-ERK or pdn-JNK (b and d), plus the empty vector pcDNA. The latter was used to ensure equal amount of total DNA in transfection assays. The amount of DNA of pa-ERK, or pa-JNK, plus pcDNA was equalized to 0.7 μ g (a and c). The amount of DNA of pdn-ERK, or pdn-JNK, plus pcDNA was equalized to 2 μ g (b and d). After overnight recovery, cells were serum-starved in DMEM with 0.5% DMEM for 24 h prior to the stimulation with FBS (10%) in the presence or absence of curcumin (20 μ M) for an additional 24 h. Luciferase activities were expressed as relative units after β -galactosidase normalization ($n \geq 6$). * $P < 0.05$ vs cells with no treatment (the corresponding first column on the left). † $P < 0.05$ vs cells with curcumin only, without co-transfected pa-cDNA (the corresponding third column on the left). The floating schema denotes the pPDGF- β R-Luc, or pEGFR-Luc, luciferase reporter construct in use and the application of a cDNA expressing plasmid to the system.

deplete cellular GSH,³⁰ for 1 h. As shown in Figure 8a, in luciferase assays NAC, like curcumin, significantly reduced luciferase activity in HSCs transfected with pPDGF- β R-Luc, or pEGFR-Luc. Pre-exposure of cells to BSO apparently diminished this inhibitory effect (the last two columns on the right side). This result suggested that *de novo* synthesis of GSH might reduce *pdgf- β r* and *egfr* promoter activity in HSCs. Real-time PCR (Figure 8b) and Western blotting analyses (Figure 8c) further demonstrated that NAC, like curcumin, reduced the mRNA levels and the protein abundance of PDGF- β R and EGFR in activated HSCs. Pretreatment of cells with BSO abolished this inhibitory activity (the last two columns and wells on the right side of Figure 8b and c). Taken together, these results demonstrated that *de novo* synthesis of GSH played a critical role in curcumin inhibition of *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*.

Activation of PPAR γ Induced *gclc* and *gclm* Expression in Activated HSCs *In Vitro*

We previously demonstrated that curcumin induced gene expression of PPAR γ and enhanced its activity in HSCs.¹¹ In addition, synthesis of GSH was required for curcumin suppression of *pdgf- β r* and *egfr* expression in activated HSCs

in vitro (Figure 8). To understand the underlying mechanisms, we assumed that the enhancement of PPAR γ activity by curcumin might stimulate gene expression of the catalytic (GCLc) and modifier (GCLm) subunits of GCL, which could stimulate the synthesis of GSH and ultimately lead to suppression of *pdgf- β r* and *egfr* expression. To test this assumption, passaged HSCs were pretreated with or without PD68235 (20 μ M), a specific PPAR γ antagonist, for 30 min prior to addition of curcumin (20 μ M) for an additional 24 h. As shown in Figure 9a, in Western blotting analyses, curcumin enhanced, as expected, the abundance of GCLc and GCLm in passaged HSCs (Zheng,²¹ no. 7416). Pretreatment with the PPAR γ antagonist, PD68235, dramatically abrogated the stimulatory effect, suggesting that the process might require activation of PPAR γ . To verify the role of PPAR γ activation in the induction of *gclc* and *gclm* expression, passaged HSCs were stimulated with the natural PPAR γ agonist, PGJ₂, at various concentrations for 24 h. Results from real-time-PCR (Figure 9b) and Western blotting analyses (Figure 9c) confirmed that activation of PPAR γ by PGL₂ induced *gclc* and *gclm* expression in a dose-dependent manner. Taken together, these results revealed that activation of PPAR γ induced *gclc* and *gclm* expression in activated HSCs *in vitro*.

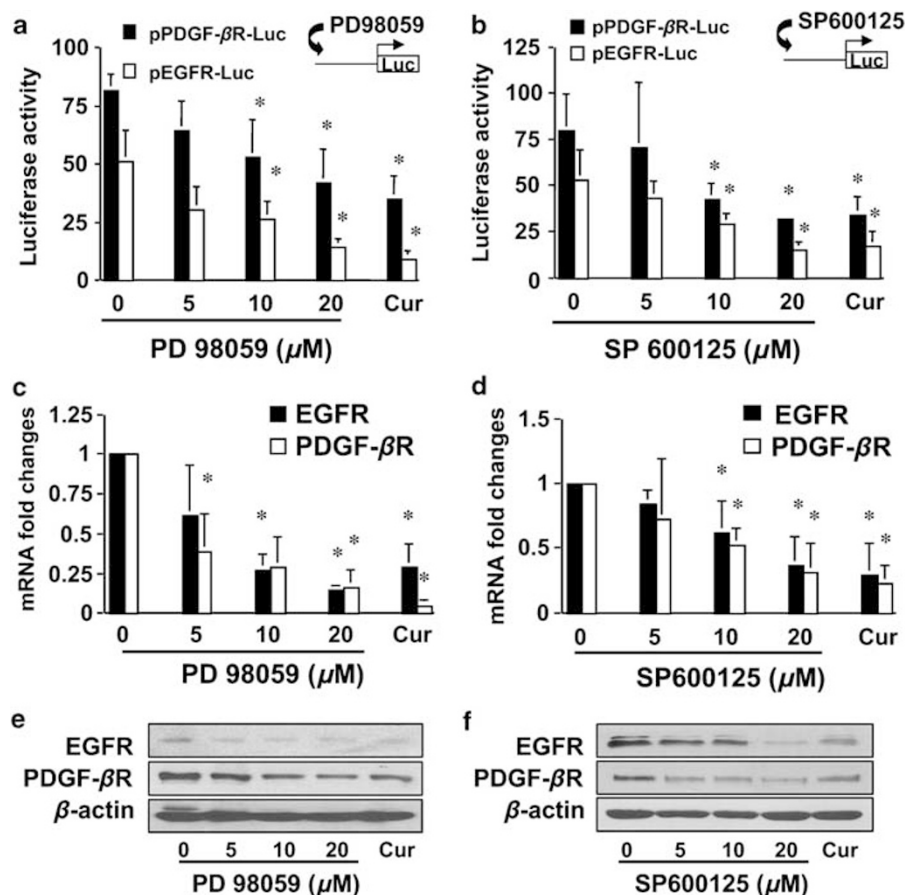


Figure 7 The inhibition of ERK or JNK activity reduced *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*. Cultured HSCs in DMEM with 10% of FBS were treated with curcumin (20 μ M), or the ERK inhibitor PD98059 (**a**, **c**, and **e**), or the JNK inhibitor SP600125 (**b**, **d**, and **f**), at the indicated doses for 24 h. (**a** and **b**). Luciferase assays of cells transfected with pPDGF- β -Luc, or pEGFR-Luc. Luciferase activities were expressed as relative units after β -galactosidase normalization ($n = 6$). * $P < 0.05$ vs the un-treated control (the corresponding first column on the left). The floating schema denotes the pPDGF- β -Luc, or pEGFR-Luc, luciferase reporter construct in use and the application of a kinase inhibitor to the system; (**c** and **d**) real-time PCR analyses of the mRNA levels of PDGF- β R or EGFR. GAPDH was used as an invariant control for calculating fold changes of target mRNA ($n = 3$). * $P < 0.05$ vs the untreated control (the corresponding first column on the left); (**e** and **f**) Western blotting analyses of the abundance of PDGF- β R or EGFR. β -Actin was used as an invariant control for equal loading. Representative result is shown from three independent experiments.

DISCUSSION

We previously proposed that there might exist an antagonistic relationship between activation of the signaling pathways for growth factors, including PDGF and EGF, and activation of PPAR γ in HSC, that is, activation of the signaling pathways for PDGF and EGF reduces the activity of PPAR γ by suppressing PPAR γ gene expression during HSC activation, whereas activation of PPAR γ results in interruption of the signaling pathways, leading to inhibition of HSC activation.^{20,22} Our prior results supported the proposal and demonstrated that activation of PDGF or EGF signaling suppressed gene expression of PPAR γ in activated HSCs *in vitro*. The current study was aimed at elucidating the effect of PPAR γ activation on the PDGF or EGF signaling in HSC, and at elucidating the underlying mechanisms. In this report, we demonstrated that activation of PPAR γ by curcumin or its agonist interrupted the PDGF or EGF signaling pathways by reducing the phosphorylation levels of PDGF- β R and EGFR,

and suppressing *pdgf- β r* and *egfr* expression. Further experiments revealed that activation of PPAR γ induced expression of GCL genes and stimulated synthesis of GSH, leading to suppression of *pdgf- β r* and *egfr* expression.

The tyrosine kinase receptors of EGFR and PDGFR have received a great deal of attention as potential therapeutic targets for treatment of hepatic fibrosis,^{31,32} as these receptors have been shown to play key roles in hepatic fibrogenesis.³⁻⁶ As expected, exogenous PDGF and EGF stimulated cell proliferation of HSC in a dose-dependent manner, which was diminished by curcumin (Figure 1). However, different strategies of treatment with curcumin, that is, pretreatment or simultaneous treatment, showed different inhibitory efficiencies. Pretreatment with curcumin strengthened the inhibitory effect on cell proliferation. This discrepancy in the inhibitory efficiencies indicated that the inhibitory effect of curcumin on PDGF- or EGF-stimulated cell proliferation might not result from direct interactions between curcumin

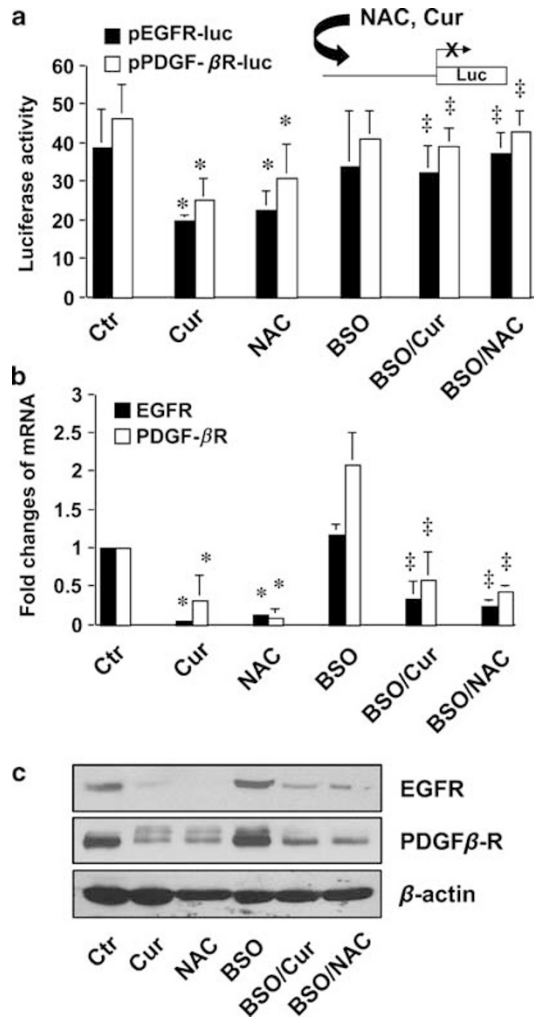


Figure 8 The inhibition of *de novo* GSH synthesis eliminated the inhibitory effect of curcumin on *pdgf- β r* and *egfr* expression in activated HSCs *in vitro*. HSCs in DMEM with 10% of FBS were treated for 24 h with curcumin (20 μ M) or NAC (5 mM) with or without the pre-exposure to BSO (0.25 mM) for 1 h. Values were means \pm s.d. ($n \geq 3$). * $P < 0.05$, vs cells with no treatment (the corresponding first column on the left); $^{\ddagger}P < 0.05$, vs cells treated with curcumin, or NAC, only (the corresponding second or the third column on the left). (a) Luciferase assays of cells transfected with pPDGF- β R-Luc, or pEGFR-Luc. Luciferase activities were expressed as relative units after β -galactosidase normalization ($n = 6$). The floating schema denotes the pPDGF- β R-Luc, or pEGFR-Luc, luciferase reporter construct in use and the application of NAC or curcumin to the system; (b) real-time PCR analyses of the mRNA levels of PDGF- β R, or EGFR. GAPDH was used as an invariant control for calculating fold changes of target mRNA ($n = 3$); (c) Western blotting analyses of the abundance of PDGF- β R, or EGFR. β -Actin was used as an invariant control for equal loading. Representative result is shown from three independent experiments.

and PDGF or EGF. Further experiments revealed that curcumin reduced the abundance of PDGF- β R and EGFR in activated HSCs after a 24-h treatment (Figures 2–4). In addition, curcumin significantly and rapidly reduced phosphorylation levels of PDGF- β R and EGFR (Figure 5), which reached its peak within 10–20 min. These observations could partially explain the discrepancy in the inhibitory efficiencies.

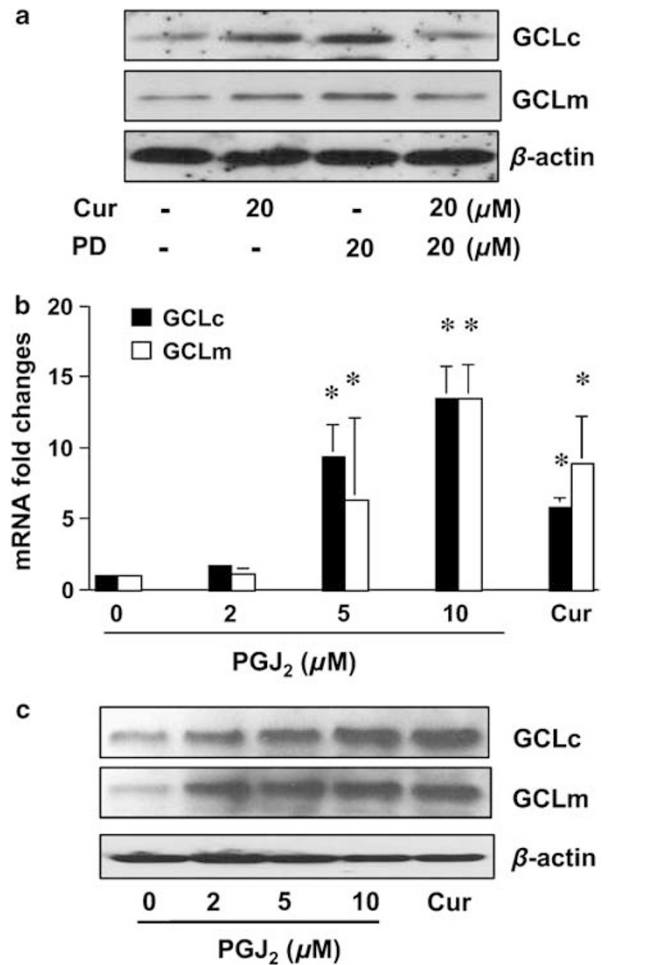


Figure 9 The activation of PPAR γ induced *gclc* and *gclm* expression in activated HSCs *in vitro*. (a) Cultured HSCs in DMEM with 10% of FBS were pretreated with or without the PD68235 (20 μ M), a specific PPAR γ antagonist, for 1 h prior to the addition of curcumin at the indicated concentrations for an additional 24 h. Western blotting analyses of GCLc and GCLm. β -Actin was used as an invariant control for equal loading. Representative was shown from three independent experiments. (b and c) Cultured HSCs in DMEM with 10% of FBS were treated with curcumin (20 μ M) or PGJ $_2$ at the indicated concentrations for 24 h. (b) Real-time PCR analyses of the mRNA levels of GCLc and GCLm. GAPDH was used as an invariant control for calculating fold changes of target mRNA ($n = 3$). * $P < 0.05$ vs the untreated control (the corresponding first column on the left); (c) Western blotting analyses of the abundance of GCLc and GCLm. β -Actin was used as an invariant control for equal loading. Representative result is shown from three independent experiments.

The activation of PDGF and EGF signaling was receptor-dependent. Simultaneous treatment with curcumin rapidly interrupted PDGF and EGF signaling by blocking tyrosine phosphorylation of PDGF- β R and EGFR. It was an instantaneous, but short-term, action. The interruption of PDGF and EGF signaling by suppression of *pdgf- β r* and *egfr* expression might require much longer time, such as several hours. Therefore, PDGF and EGF could activate their signaling and stimulate cell proliferation during the period of time after the peak of the dephosphorylation of the receptors and before

the significant reduction in the abundance of the receptors. Therefore, in the case of simultaneous treatment with curcumin plus PDGF or EGF, the stimulatory effects of PDGF and EGF on cell proliferation were delayed and attenuated, but still executed. In great contrast, pretreatment with curcumin for 24 h resulted in significant reduction in the abundance of PDGF- β R and EGFR. In this case, there might not be enough PDGF- β R and EGFR for binding to PDGF and EGF to initiate their signaling at the very beginning. Therefore, pretreatment with curcumin could result in a more efficient inhibitory effect on cell proliferation. It bears notice that the serum-depleted medium was used in these experiments to eliminate the influence of unidentified mitogenic compounds present in serum or serum-derived medium additives. In addition, serum rich media, for example, media with 10% FBS, contain enough growth factors, including PDGF and EGF, for stimulating cell proliferation, which could interfere with and attenuate the stimulatory effects of exogenous PDGF or EGF.³³

Recent studies have demonstrated that pro-adipogenic factors inhibit HSC activation.³⁴ Adipocyte differentiation is positively stimulated by PPAR γ ,^{35,36} and negatively regulated by PDGF and EGF.^{37,38} PPAR γ stimulated adipogenesis and inhibited HSC activation.^{34–36} Adipogenic regulation might be critical for HSCs to maintain and restore the quiescent phenotypes.³⁴ To determine the relationship between PPAR γ activation and the growth factors in regulating adipogenesis, it was previously demonstrated that PPAR γ transcriptional activity was reduced by PDGF through phosphorylation of PPAR γ by MAP kinases.³⁹ We recently reported that PDGF and EGF suppressed gene expression of PPAR γ in cultured HSCs.²² The interruption of the PDGF and EGF signaling pathways by curcumin stimulated gene expression of PPAR γ in HSC *in vitro*.²² The inhibition of ERK and JNK activity played a critical role in the induction of gene expression of PPAR γ in HSCs.²² On the other hand, our preliminary results suggested that activation of PPAR γ might, in turn, inhibit ERK and JNK activity in cultured HSCs (data not shown here). The current studies further evaluated the relationship between PPAR γ activation and PDGF and EGF signaling in activated HSCs *in vitro*. Our results indicated that activation of PPAR γ interrupted PDGF and EGF signaling by reducing the level of the phosphorylation of PDGF- β R and EGFR, and by suppressing gene expression of the receptors. Our results collectively demonstrated the existence of the antagonistic relationship between activation of PPAR γ and stimulation of PDGF and EGF signaling in HSCs.

Curcumin has been studied as a therapeutic or preventive candidate because of its antioxidative and anti-inflammatory properties.^{17,40} In phase I clinical studies, curcumin with doses up to 3600–8000 mg daily for 4 months did not result in discernible toxicities, except mild nausea and diarrhea.⁴¹ The cytotoxicity of curcumin to cultured HSCs was previously evaluated.¹¹ 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 μ M was not toxic to cultured HSCs. The working concentration of curcumin used in this study was 20 μ M, which is in the range of concentrations used by most of others. This concentration is much higher than those observed in blood and/or tissues of human and animals.^{42,43} Systemic bioavailability of curcumin is relatively low.⁴⁴ Curcumin concentrations in human plasma could reach up to 2 μ M after oral intake of very high amounts of curcumin.⁴⁵ Although higher concentrations of curcumin in blood might be possible, depending on the actual composition of food,⁴⁶ most pharmacokinetic studies in humans point out that only low micromolar levels of curcumin can be found in blood. We recently observed that oral administration of curcumin at 200 mg/kg body weight significantly reduced the levels of the transcript and protein of PDGF- β R and EGFR in the liver, inhibited HSC activation, and protected the liver from injury caused by CCL₄.¹⁵ It bears emphasis that because the *in vivo* system is multi-factorial, directly extrapolating *in vitro* conditions and results, for example, effective concentrations, to the *in vivo* system might be misleading.

Increasing evidence has demonstrated that oxidative stress promotes HSC activation and collagen production and plays an important role in the pathogenesis of liver fibrosis.^{47–49} Mammalian cells respond to oxidative stress through antioxidant defense, which includes antioxidant enzymes and non-enzyme molecules. GSH is the predominant low-molecular-weight thiol and the most important non-enzyme antioxidant. GSH effectively protects cells against damage caused by oxidative stress, which might prevent diseases associated with oxidative stress.⁵⁰ We previously showed that curcumin elevated the level of cellular GSH in cultured HSC by increasing GCL activity through induction of expression of the two subunits of GCL.²¹ We further demonstrated in this report that blockade of the synthesis of GSH attenuated the effects of curcumin on the suppression of *pdgf- β r* and *egfr* expression in HSCs (Figure 8). The process was mediated by activation of PPAR γ through induction of gene expression of GCL (Figure 9). Our results are consistent with other prior observations. In many cell types, gene expression of PDGF- β R and EGFR is associated with increase in oxidative stress, which might represent pathophysiological determinants.^{51–53} Additional experiments are necessary to elucidate the underlying mechanisms by which oxidative stress regulates *pdgf- β r* and *egfr* expression in HSCs.

Based on prior and current observations, a simplified model is proposed in Figure 10 to describe the mechanisms by which the curcumin activation of PPAR γ interrupts PDGF and EGF signaling. Curcumin rapidly interrupts PDGF- β R and EGFR signaling in activated HSCs by reducing the phosphorylation levels of PDGF- β R and EGFR, as well as of their downstream signaling inter-mediators ERK and JNK. This instant action causally stimulates gene expression²² and activity of PPAR γ ,³⁹ which critically lead to attenuation of oxidative stress by inducing gene expression of GCL and

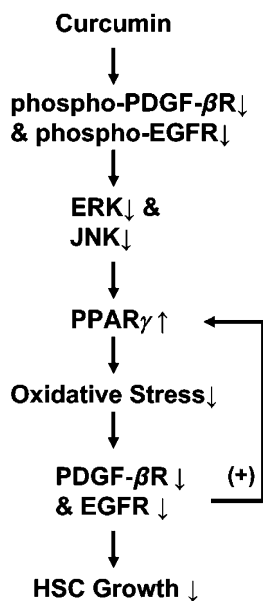


Figure 10 Schema of the underlying mechanism of curcumin in the interruption of PDGF and EGF signaling in activated HSC *in vitro*. Curcumin rapidly interrupts PDGF- β R and EGFR signaling in activated HSCs by reducing the phosphorylation levels of PDGF- β R and EGFR, as well as of their downstream signaling inter-mediators ERK and JNK. This instant action causally stimulates gene expression and its activity of PPAR γ , which critically leads to attenuation of oxidative stress by induction of gene expression of GCL and elevation of the level of cellular GSH. The actions of curcumin, including activation of PPAR γ and elevation of GSH contents, collectively result in suppression of *pdgf- β r* and *egfr* expression, leading to long-term interruption of PDGF- β R and EGFR signaling and inhibition of cell proliferation.

elevating the level of cellular GSH. The actions of curcumin, including activation of PPAR γ and elevation of GSH contents, collectively result in suppression of *pdgf- β r* and *egfr* expression, leading to long-term interruption of PDGF- β R and EGFR signaling, and inhibition of cell proliferation. In addition, interruption of signaling for PDGF and EGF antagonistically induces gene expression of PPAR γ .²² It bears emphasis that the underlying mechanisms are certainly more complex than what described here. It remains unclear what factors are involved in the instant curcumin reduction of the phosphorylation levels of PDGF- β R and EGFR, and what transcription factors mediate suppression of *pdgf- β r* and *egfr* expression in HSC. In addition, our results do not exclude possible involvement of any other mechanisms in the curcumin interruption of PDGF and EGF signaling. In summary, these results support our original hypothesis that enhancement of PPAR γ activity by curcumin might result in interruption of the signaling pathways for PDGF and EGF. These results provide novel insights into the mechanisms of curcumin in the inhibition of cell proliferation and activation of HSCs.

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