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The interruption of the PDGF and EGF signaling pathways by curcumin stimulates gene expression of PPARγ in rat activated hepatic stellate cell *in vitro*

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Activation of hepatic stellate cells (HSC), the major effector in hepatic fibrogenesis, is coupled with sequential alterations in expression of genes, including the upregulation of platelet-derived growth factor-β receptor (PDGF-βR) and epidermal growth factor receptor (EGFR), as well as the down-regulation of the peroxisome proliferator-activated receptor-γ (PPARγ). However, the relationship among the alterations in expression of the genes and the activation of their signaling in activated HSC remains obscure. We recently showed that curcumin, the yellow pigment in curry, inhibited cell growth and induced gene expression of endogenous PPARγ in activated HSC *in vitro*. The present study is to elucidate the underlying mechanisms, focusing on the impacts of PDGF and EGF signaling. It is hypothesized that the interruption of the PDGF and EGF signaling pathways by curcumin might stimulate gene expression of PPARγ in activated HSC. Our results in this report indicate that the activation of PDGF or EGF signaling by exogenous PDGF or EGF inhibits PPARγ gene expression in passaged HSC. Curcumin interrupts PDGF and EGF signaling demonstrated by inhibiting tyrosine phosphorylation of PDGF-βR and EGFR and by reducing the levels of phosphorylated phosphatidylinositol-3 kinase (PI-3K/AKT), extracellular signal-regulated kinase (ERK) and the Jun N-terminal kinase (JNK). The blockade of PI-3K/AKT, ERK or JNK signaling negatively regulates PPARγ gene expression in activated HSC, leading to the reduction in cell growth, including inducing cell arrest and apoptosis. Our results collectively demonstrate that the interruption of the PDGF and EGF signaling pathways by curcumin stimulates gene expression of PPARγ in activated HSC. These results provide novel insights into the mechanisms of curcumin in the induction of PPARγ gene expression in activated HSC.
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Hepatic stellate cells (HSC) are the most relevant cell type for the development of liver fibrosis.[1,2] During liver injury, HSC become activated and trans-differentiate into myofibroblast-like cells. The process is called HSC activation. Enhanced HSC proliferation is a dramatic feature during the pathogenesis of liver fibrosis. It is mainly triggered by autocrine/paracrine activation of the signaling for mitogenic platelet-derived growth factor-β (PDGF) and epidermal growth factor (EGF).[3–6] The process of HSC activation is coupled with the up-expression of receptors for PDGF and EGF,[4,7,8] as well as the dramatic down-expression of peroxisome proliferator-activated receptor-γ (PPARγ).[9–11] Both PDGF-βR and EGFR contain intrinsic tyrosine kinase activity. Upon binding to their ligands, the receptors become phosphorylated at tyrosine residues, leading to the activation of their downstream signaling, of which the mitogen-activated protein kinase (MAPK) pathways and the phosphatidylinositol 3-kinase (PI-3K) pathway are the major signaling routes.[12,13] The MAPK family includes the extracellular signal-regulated kinase (ERK) pathway and the Jun N-terminal kinase (JNK) pathway. Activated PI-3K generates several phosphoinositols, leading to AKT activation by phosphorylation at Thr^{308} and Ser^{473},[14] which is inhibited by activation of phosphatase and tensin homolog (PTEN).[13] It is well-known that these signaling pathways regulate multiple biological processes, including gene expression, cell proliferation and survival.[12,13] PPARs belong to the superfamily of nuclear receptors. PPARγ, one of the PPAR isoforms, is the most wildly studied. PPARγ controls growth and differentiation in different tissues. Although the level of PPARγ is high in quiescent HSC in

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normal liver, its expression and its activity are dramatically diminished during HSC activation *in vitro* and *in vivo*.[9–11] Activation of PPARγ induces cell arrest and apoptosis of activated HSC.[9–11] Introduction of exogenous PPARγ cDNA is sufficient to reverse the morphology of activated HSC to the quiescent phenotype.[15]

Curcumin is the yellow pigment in curry from turmeric. It has received attention as a promising dietary supplement for cancer prevention[16–18] and the protection against fibrogenic insults.[19,20] However, the underlying mechanisms remain incompletely understood.[21] We recently reported that curcumin induced gene expression of endogenous PPARγ in activated HSC *in vitro*, which was a prerequisite in the curcumin inhibition of HSC growth, induction of apoptosis and suppression of extracellular matrix gene expression.[22–24] The aim of this study is to elucidate the mechanisms of curcumin in the induction of PPARγ gene expression in activated HSC, focusing on the impact of PDGF and EGF signaling. It is hypothesized that the interruption of the PDGF and EGF signaling pathways by curcumin might stimulate gene expression of PPARγ in activated HSC. Results in this report support our hypothesis and demonstrate that activation of PDGF or EGF signaling inhibits PPARγ gene expression. Curcumin interrupts PDGF and EGF signaling, leading to the induction of gene expression of PPARγ and the reduction of cell growth of activated HSC *in vitro*.

MATERIALS AND METHODS

Materials

Curcumin (purity > 94%) was purchased from Sigma (St Louis, MO, USA) and used at 20 μM, unless indicated otherwise. PD68235, a specific PPARγ antagonist, was kindly provided by Pfizer (Ann Arbor, MI, USA).[25] The PI-3K inhibitor LY294002 and the MEK inhibitor PD98059 were purchased from CalBiochem (La Jolla, CA, USA), and the JNK inhibitor SP600125 was purchased from Sigma. Recombinant PDGF and EGF were purchased from Cell Sciences (Canton, MA, USA).

Isolation and Culture of HSC

HSC were isolated from Sprague–Dawley rats (200–250 g) as we described previously.[26] Cells were cultured in Dullbecco's modified Eagle's medium (DMEM) with 10% of fetal bovine serum (FBS). Semiconfluent HSC with 4–8 passages were used for experiments. In some of experiments, cells were serum-starved for 48 h in DMEM with 0.5% of FBS before treatment.

Western Blotting Analyses

Whole-cell extracts were prepared as we described.[26] Proteins were separated by SDS-PAGE with 10% of resolving gel, and transferred to PVDF membrane. Target proteins were respectively detected by primary antibodies against PPARγ, phosphorylated types of PDGF-βR, EGFR, ERK1/2, JNK1/2 or AKT, and the corresponding non-phosphorylated types of total proteins; Bcl-2, Bax or cyclin D1, and subsequently by horseradish peroxidase-conjugated secondary antibodies (Santa Cruze Biotechnology, Santa Cruze, CA, USA). Protein bands were visualized by using a chemiluminescence reagent (Amersham Biosci, Piscataway, NJ, USA). In Western blotting analyses, the densities of bands were normalized with the internal invariable control β-actin. The level of target protein bands was densitometrically determined by using Quantity One® 4.4.1 (Bio-Rad). The variation in the density was expressed as fold changes compared to the control in the blot.

RNA Isolation and Real-Time PCR

Total RNA was extracted by using TRI-Reagent® (Sigma), following the protocol provided by the manufacturer. Real-time PCR was carried out as described previously.[22] mRNA fold changes of target genes relative to the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control were calculated as suggested by Schmittgen *et al*.[27] The following primers were used in real-time PCR:

PPARγ:
(F) 5′-ATTCTGGCCCACCAACTTCGG-3′;
(R) 5′-TGGAAGCCTGATGCTTTATCCCCA-3′;

GAPDH:
(F) 5′-GGCAAATTCAACGGCACAGT-3′;
(R) 5′-AGATGGTGATGGGCTTCCC-3′.

Plasmid Constructs

The PPARγ promoter luciferase reporter plasmid pPPARγ-Luc contain the 5′-flanking region (−2776 bp) of the PPARγ gene promoter in a luciferase reporter plasmid.[28] It was kindly provided by Dr Johan Auwerx (Pasteur Institute, Lille, France).[28] The plasmid pdn-PTEN or pwt-PTEN contains a full-length of cDNA encoding the dominant-negative form of PTEN or wild-type PTEN, respectively. They were kindly provided by Dr Christopher G Kevil (Louisiana State University Health Sciences Center in Shreveport). The plasmid pdn-ERK or pdn-JNK, contains a full length of cDNA encoding the dominant-negative form of ERK or JNK respectively, which were described previously.[29] The plasmid pa-ERK or pa-JNK contains cDNA encoding the constitutively active form of ERK or JNK, respectively, which were described previously.[29,30]

Transient Transfection Assays

Semiconfluent HSC in six-well plastic plates were transiently transfected with a reporter plasmid (3 μg DNA/well, if no specific indication), by using LipofectAMINE® (Life Technologies), following the protocol provided by the manufacturer. Transfection efficiency was controlled by co-transfection of the β-galactosidase reporter plasmid pSV-β-gal (1 μg/well) (Promega). Luciferase activity was measured using an automated luminometer (Turners). β-Galactosidase assays were performed using an assay kit from Promega

Corp. Each treatment had a triplicate in every experiment. Each experiment was repeated at least three times. Luciferase activity was expressed as relative unit after normalization with β-galactosidase activity.

Analyses of Cell Growth

The number of viable cells was used to determine cell growth by using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega) (ie MTS assays).

Detection of Apoptotic HSC by TUNEL

Pre-confluent HSC in slideflasks were serum starved for 24 h. Cells were subsequently treated as described for an additional 24 h. Cells were washed three times with cold PBS before fixation. Apoptotic HSC were detected by DeadEnd™ Fluorometric TUNEL System (Promega), following the protocol provided by the manufacturer.

Flow Cytometric Analyses of Apoptotic HSC

Semiconfluent HSC were treated as indicated. Cells were harvested by brief trypsin/EDTA treatment and washed several times with cold PBS. HSC ($\geq 1 \times 10^6$ cells/each sample) were suspended in 2 ml of FACS buffer (1% FA buffer (Difco), 0.1% sodium azide and 1% FBS). Cells were fixed with ethanol, and then labeled with propidium iodide (PI; Sigma). Cells that were positively labeled with PI were detected by a Coulter® EPICS® XL-MCL flow cytometer. The rate of apoptosis was analyzed using its System II™ software, as described previously.[31]

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 the Dunnett's test for *post hoc* analysis.

RESULTS

Exogenous PDGF or EGF Reduces Gene Expression of PPARγ, which was Partially Eliminated by Curcumin

The process of HSC activation is coupled with the sequential upregulation of PDGF-βR and EGFR and activation of their signaling, as well as the dramatic reduction in expression of PPARγ.[4,7–11] Prior other studies have suggested the opposite functions of PDGF and PPARγ in adipogenesis.[32,33] To evaluate the effect of activation of PDGF and EGF signaling on regulation of gene expression of PPARγ, exogenous PDGF and EGF were added to passaged HSC for an additional 24 h after the treatment with or without curcumin in serum-free media for 24 h. PPARγ expression was determined by real-time PCR and Western blotting analyses. Passaged HSC cultured in serum-depleted media respond better to the stimulation of PDGF or EGF.[4,31] As shown in Figure 1, exogenous PDGF or EGF evidently inhibited PPARγ expression at both levels of mRNA and protein in a dose-dependent manner. Compared with the control with no treatment (the first black column on the left), EGF at 80 ng/ml or PDGF at 30 ng/ml reduced the steady-state level of PPARγ mRNA by 66 and 75%, respectively (Figure 1a and c). The inhibitory effects of EGF and PDGF on the abundance of PPARγ were verified by Western blotting analyses (Figure 1b and d). However, if cells were preexposed to curcumin (20 μM), the inhibitory effect of EGF or PDGF was significantly reduced (Figure 1). For instance, compared with cells exposed to EGF at 80 ng/ml or PDGF at 30 ng/ml, the pre-exposure of cells to curcumin partially but significantly eliminated the reduction caused by EGF or PDGF, and increased the level of PPARγ mRNA by 2.4- and 2.25- fold, respectively (Figure 1a and c). This observation was verified by Western blotting analyses. In every pair of cells treated with the same dose of EGF (Figure 1b) or PDGF (Figure 1d), the pre-exposure of cells to curcumin significantly increased the level of PPARγ. These data collectively suggested that the activation of PDGF or EGF signaling by exogenous PDGF or EGF might suppress

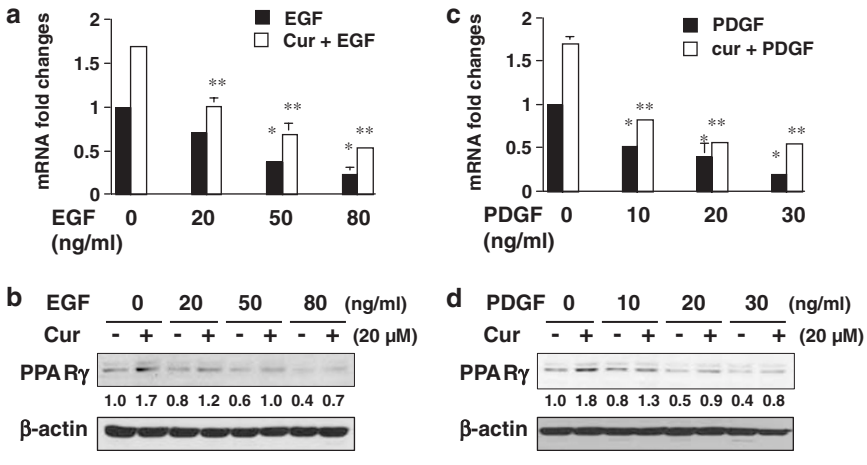

Figure 1 Exogenous EGF or PDGF dose-dependently reduces gene expression of PPARγ in activated HSC *in vitro*. After the treatment with or without curcumin in serum-free media for 24 h, passaged HSC were stimulated with PDGF or EGF at indicated doses in serum-free media for an additional 24 h. Total RNA or protein extracts were prepared from the cells for real-time PCR (**a** and **c**) ($n=3$), or Western blotting analyses (**b** and **d**) ($n=3$). β-Actin was used as an internal invariable control for (**b** and **d**). The level of PPARγ was densitometrically normalized with β-actin. The numbers beneath the blots were fold changes in the densities of PPARγ bands compared to the control without treatment in the blot ($n=3$). Because of the limited space, standard deviations were not presented. *$P<0.05$ *vs* cells with no treatment (The first black column on the left). **$P<0.05$ *vs* cells with only EGE or PDGF at the same dose.

PPARγ expression, which could be partially eliminated by curcumin.

Curcumin Reduces Tyrosine Phosphorylation of PDGF-βR and EGFR

Additional experiments were performed to elucidate the effect of curcumin on tyrosine phosphorylation of PDGF-βR and EGFR in activated HSC, which might lead to interruption of the signaling pathways of PDGF and EGF and inactivation of downstream MAPK and PI-3K/AKT. Pilot studies indicated that the peak of tyrosine phosphorylation of PDGF-βR or EGFR was within 15–20 min after the addition of FBS, which contains significant amounts of PDGF and EGF[4,31] and that the process lasted no less than 60 min (data not shown). Cells were serum-starved in DMEM for 48 h, which made cells more sensitive to serum stimulation.[22–24] Serum-starved cells were pretreated with curcumin for 30 min before the stimulation with FBS (10%) for an additional 20 min. Whole-cell extracts were prepared. Phosphorylated PDGF-βR and EGFR were detected by Western blotting analyses using antibodies against phospho-PDGF-βR (1:2000) and EGFR (1:500), respectively. As shown in Figure 2, curcumin significantly and dose-dependently reduced tyrosine phosphorylation of PDFG-βR (Figure 2a) and EGFR (Figure 2b) in HSC. As controls, the levels of total PDFG-βR (Figure 2a) and EGFR (Figure 2b) were not changed. These results suggested that curcumin might interrupt PDGF and EGF signaling by reducing receptor tyrosine phosphorylation in passaged HSC.

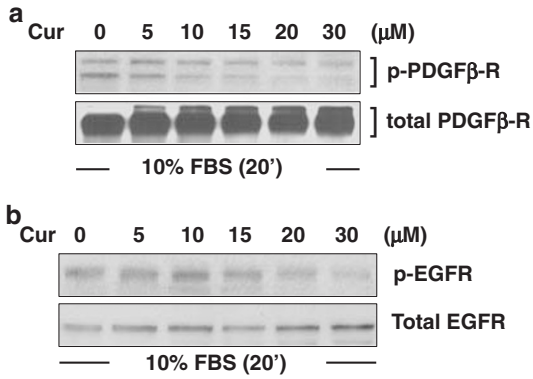

Figure 2 Curcumin dose-dependently reduces tyrosine phosphorylation of PDGF-βR and EGFR in activated HSC *in vitro*. After serum-starvation for 48 h, HSC were pretreated with curcumin for 30 min before the stimulation with FBS (10%) for an additional 20 min. Whole-cell extracts were prepared for Western blotting analyses. Representatives were shown here from three independent experiments. (**a**) Curcumin dose-dependently reduces the level of phosphorylatetd PDGF-βR (p-PDGF-βR). Total PDGF-βR was used as an internal control. (**b**) Curcumin dose-dependently reduces the level of phosphorylatetd EGFR (p-EGFR). Total EGFR was used as an internal control.

Curcumin Blocks the Activation of the ERK, JNK and PI-3K/AKT Signaling Pathways in Activated HSC

The ability of curcumin to interrupt PDGF and EGF signaling was verified by evaluating the effects of curcumin on the activity of ERK, JNK and AKT. Pilot studies indicated that the peak of tyrosine phosphorylation of PDGF-βR or EGFR was within 15–20 min after the stimulation with FBS (data not shown). Serum-starved HSC were pretreated with curcumin for 30 min before the stimulation with FBS (10%) for an additional 20 min. Whole-cell extracts were prepared. As shown in Figure 3 by Western blotting analyses, curcumin dose-dependently reduced the level of phosphorylated ERK (Figure 3a), JNK (Figure 3b) and AKT (Figure 3c). In great contrast, the phyto-chemical had no impact on the level of total ERK, JNK and AKT (Figure 3). These results suggested that curcumin might block the activation of the ERK, JNK and PI-3K/AKT signaling pathways in passaged HSC, which confirmed the effect of curcumin on the interruption of PDGF and EGF signaling in HSC.

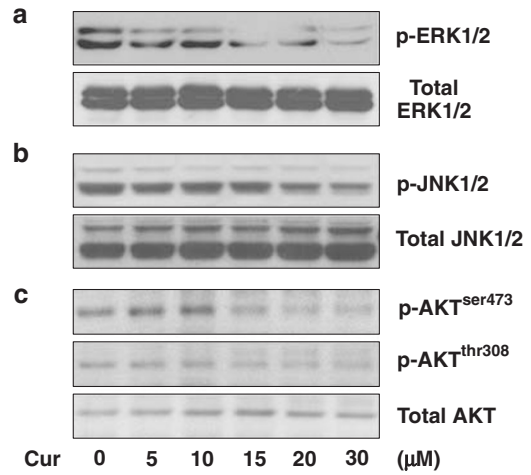

Figure 3 Curcumin blocks the activation of the ERK, JNK and PI-3K/AKT signaling pathways in activated HSC *in vitro*. After serum-starvation for 48 h, HSC were pre-treated with curcumin for 30 min before the stimulation with FBS (10%) for an additional 20 min. Whole cell extracts were prepared for Western blotting analyses. Representatives were shown from three independent experiments. (**a**) Curcumin reduces the level of phosphorylatetd ERK1/2 (p-ERK1/2). Total ERK1/2 was used as an internal control. (**b**) Curcumin reduces the level of phosphorylatetd JNK1/2 (p-JNK1/2). Total JNK1/2 was used as an internal control for equal loading. (**c**) Curcumin reduces the level of phosphorylatetd AKT (p-AKT^{ser473} and p-AKT^{thr308}). Total AKT was used as an internal control.

Alterations in ERK, JNK or PI-3K/AKT Activity Change the Promoter Activity of PPARγ Gene in Activated HSC

Our results suggested that the activation of PDGF and EGF signaling might suppress gene expression of PPARγ in activated HSC (Figure 1). It was, therefore, assumed that stimulation or inhibition of the activity of the downstream inter-mediators of the signaling, for example, ERK, JNK or PI-3K/AKT, might respectively result in the reduction or induction of gene expression of PPARγ in HSC. To test the assumption, HSC were co-transfected with pPPARγ-Luc plus a cDNA expressing plasmid of pa-ERK, pa-JNK or

pwt-PTEN, or a plasmid of pdn-ERK, pdn-JNK or pdn-PTEN. The plasmid pPPARγ-Luc is a PPARγ promoter luciferase reporter plasmid, which contains the 5′-flanking region (−2776 bp) of the PPARγ gene promoter subcloned in a luciferase reporter plasmid.[34] The cDNA expression plasmid pa-ERK, or pa-JNK, contains a fragment of cDNA encoding the constitutively active form of ERK (a-ERK), or JNK (a-JNK), respectively. The plasmid pwt-PTEN contains a full size of wild-type PTEN cDNA, which inhibits activation of PI-3K/AKT signaling.[35] Similarly, the plasmid pdn-ERK, pdn-JNK or pdn-PTEN contains a fragment of cDNA encoding the dominant-negative form of ERK (dn-ERK), JNK (dn-JNK) or PTEN (dn-PTEN), respectively. 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 pPPARγ-Luc, 0.5 μg of pSV-β-gal, and 0.7 μg (Figure 4a and d), or 2 μg (Figure 4b and c) of a cDNA-expressing plasmid at indicated doses and the empty vector pcDNA. The latter was used to ensure an equal amount of total DNA in transfection assays. After transfection, cells were serum-starved in DMEM for 24 h before the stimulation with FBS (10%) in the presence or absence of curcumin (20 μM) for an additional 24 h. As shown in Figure 4 by luciferase assays, compared with no treatment (the first columns on the left), the activation of ERK, JNK or PI-3K/AKT, respectively, by pa-ERK, pa-JNK (Figure 4a), or by pdn-PTEN (Figure 4c), significantly reduced luciferase activity. These results suggested that the activation of ERK, JNK or PI-3K/AKT signaling might inhibit the gene promoter activity of PPARγ in HSC. Curcumin, as expected, dramatically stimulated the promoter activity of PPARγ demonstrated by the increase in luciferase activity (the third columns on the left in Figure 4a and c). It was of interest to observe that the activation of ERK, JNK, or PI-3K/AKT, by pa-ERK, pa-JNK (Figure 4a), or by pdn-PTEN (Figure 4c), dose-dependently eliminated the stimulatory effect of curcumin on luciferase activity. In great contrast, the blockade of these signaling pathways by pdn-ERK, pdn-JNK (Figure 4b), or by pwt-PTEN (Figure 4d) caused a dose-dependent increase in luciferase activity. Wild-type PTEN acts as an 'off' switch for PI-3K signaling.[35] These results collectively suggested that the alterations in the activity of ERK, JNK or PI-3K/AKT might negatively regulate the promoter activity of PPARγ gene, leading to changes in gene expression of PPARγ in HSC.

The Inhibition of ERK, JNK or PI-3K/AKT Activity Stimulates Gene Expression of PPARγ in Activated HSC

Chemicals LY294002 (LY), PD98059 (PD) and SP600125 (SP) are commonly used for specifically inhibiting the activity of PI-3K/AKT, ERK and JNK, respectively. To further test the assumption that the alteration of the activity of PDGF and EGF signaling might negatively regulate expression of PPARγ gene in HSC, these chemicals were respectively used to selectively inhibit ERK, JNK or PI-3K. Semiconfluent HSC were transfected with the PPARγ promoter luciferase reporter plasmid pPPARγ-Luc. After recovery overnight, cells were serum-starved for 24 h in DMEM with 0.5% of FBS. Cells were pretreated with PD98059, SP600125 or LY294002 at various concentrations, or with curcumin at 20 μM, for 30 min before the stimulation with FBS (10%) for an

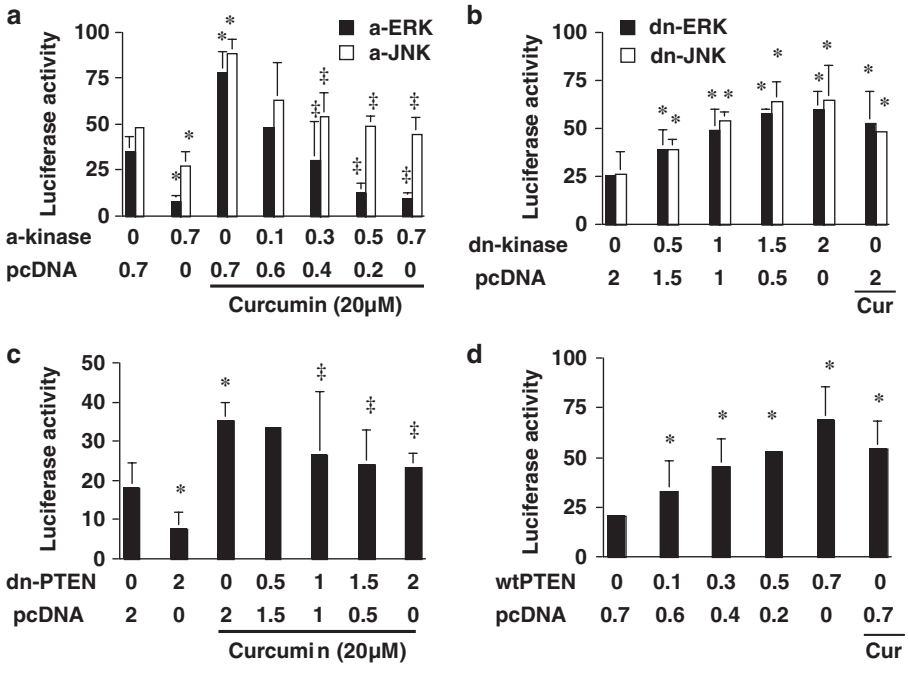

Figure 4 Alterations in ERK, JNK or PI-3K/AKT activity change the promoter activity of PPARγ gene in activated HSC. HSC in six-well plates were co-tranfected with a fixed amount of a DNA mixture per well. It includes 2 μg of pPPARγ-Luc, 0.5 μg pSV-β-gal and a plasmid expressing the active form of a kinase, that is, pa-ERK, pa-JNK, or pwt-PTEN, or a plasmid expressing the dominant negative form of a kinase, that is, pdn-ERK, pdn-JNK, or pdn-PTEN, plus the empty vector pcDNA. The latter was used to ensure the equal amount of total DNA in transfection assays. The amount of DNA of pa-ERK, pa-JNK, or pwt-PTEN plus pcDNA was equalized to 0.7 μg (**a** and **d**). The amount of DNA of pdn-ERK, pdn-JNK, or pdn-PTEN plus pcDNA was equalized to 2 μg (**b** and **c**). After recovery overnight, cells were serum-starved in DMEM for 24 h before the stimulation with FBS (10%) in the presence or absence of curcumin (20 μM) 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). $^{\ddagger}P < 0.05$ *vs* cells with curcumin only, without co-transfected pa-kinase or pdn-PTEN (the corresponding third column on the left).

additional 24 h. As shown earlier in Figures 1 and 2, FBS (10%) contained enough PDGF and EGF to activate their signaling in serum-starved HSC. Luciferase assays in Figure 5a demonstrated that each of the three specific inhibitors, like curcumin, caused a dose-dependent increase in luciferase activity, suggesting that the inhibition of ERK, JNK or PI-3K might stimulate the activity of the promoter of PPARγ gene in passaged HSC.

To confirm the observation, serum-starved HSC were pretreated with LY294002 (LY), PD98059 (PD), or SP600125 (SP), at various concentrations, or with curcumin (Cur) at 20 μM, for 30 min before the stimulation with FBS (10%) for an additional 24 h. Gene expression of PPARγ was evaluated by real-time PCR (Figure 5b) and Western blotting analyses (Figure 5c). As shown in Figure 5b and c, compared with the control with no treatment (the second columns, or wells on the left), curcumin, as expected, significantly increased gene expression of PPARγ (the first columns, or wells on the left). It was further observed that the treatment of HSC with each of the inhibitors led to a dose-dependent increase in PPARγ expression at the levels of mRNA (Figure 5b) and protein (Figure 5c). Taken together, these results indicated that the inhibition of ERK, JNK or PI-3K/AKT activity stimulated gene expression of PPARγ, which supported the assumption that alteration of the activity of PDGF and EGF signaling might negatively regulate the expression of PPARγ gene in HSC.

The Blockade of the PI-3K/AKT, ERK, and/or JNK Signaling Pathways Results in the Reduction in HSC Growth, which is Partially Eliminated by Inhibition of PPARγ Activation

Substantial evidence has indicated that the activation of PI-3K/AKT, JNK and/or ERK stimulates HSC proliferation.[6,36,37] We have previously demonstrated that the activation of PPARγ was required for curcumin to inhibit HSC proliferation.[23] Our earlier study showed that curcumin inhibited PI-3K/AKT, ERK, and JNK activity (Figure 3) and that the interruption of the signaling pathways for PI-3K/AKT, ERK and JNK induced gene expression of PPARγ (Figures 4 and 5). Prior experiments suggested that 10% of FBS might contain enough agonists to activate PPARγ in HSC.[11,22–24] The induction of PPARγ expression resulted in the stimulation of its activity in cultured HSC.[22–24] It is of interest to evaluate the role of PI-3K/AKT, ERK, and JNK activity in the curcumin induction of gene expression of PPARγ. Serum-starved HSC were divided into two groups. One group was treated with curcumin (20 μM), LY294002 (LY), PD98059 (PD) or SP600125 (SP) at the indicated concentrations, or with a mixture of the three inhibitors at the indicated concentrations in DMEM with FBS (10%) for 24 h. The other group was pretreated with the specific PPARγ inhibitor PD68235 (PD′) at 20 μM for 30 min before the addition of curcumin (20 μM), or each of the above-specific inhibitors, or the mixture of the inhibitors, at the indicated concentrations in the media with FBS (10%) for an additional 24 h. Cell growth was determined by numbers of viable cells using MTS assays. Results were expressed as changes in viable cells, compared to the cells with no treatment. As shown in Table 1, for the first group, which had no pretreatment to inhibit the

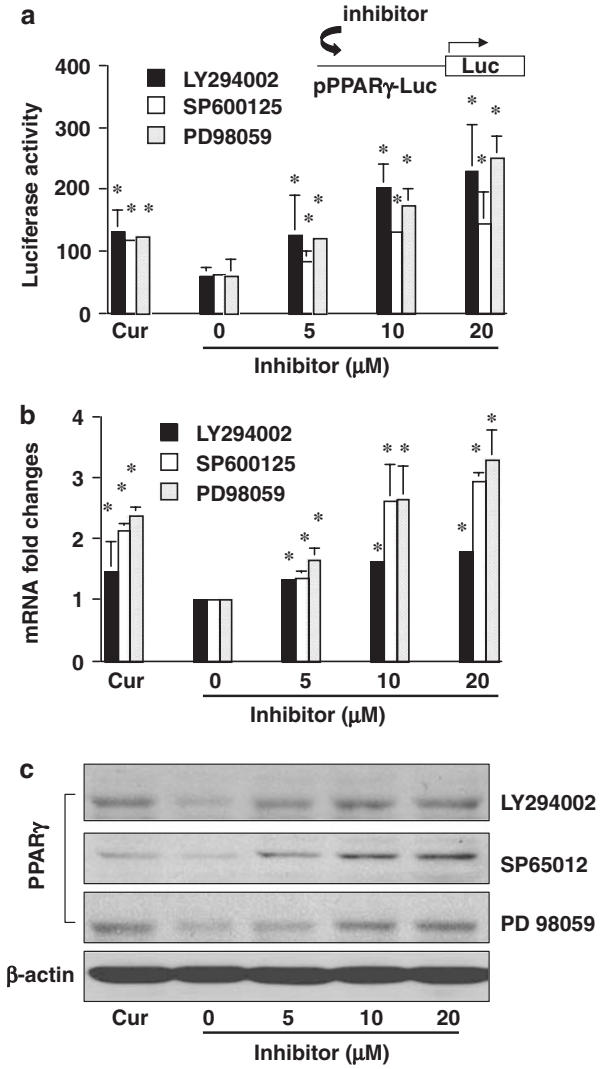

Figure 5 The inhibition of ERK, JNK or PI-3K/AKT activity stimulates gene expression of PPARγ in activated HSC. With or without transfection, serum-starved HSC were respectively pretreated with or without LY294002 (a PI-3K inhibitor), SP600125 (a JNK inhibitor), or PD98059 (a MEK inhibitor) at indicated doses, or with curcumin at 20 μM, for 30 min before the stimulation with FBS (10%) for an additional 24 h. (**a**) Luciferase assays of cells transfected with pPPARγ-Luc, containing a fragment of PPARγ gene promoter in a luciferase reporter plasmid. Luciferase activities were expressed as relative units after β-galactosidase normalization ($n \geq 6$). The floating schema denotes the pPPARγ-Luc luciferase reporter construct in use and the application of an inhibitor to the system. *$P < 0.05$ *vs* cells with no treatment (the second columns on the left). (**b**) Real-time PCR analyses of PPARγ mRNA ($n = 3$). *$P < 0.05$ *vs* cells with no treatment (the second columns on the left); (**c**) Western blotting analyses of PPARγ. Representatives were shown here from three independent experiments.

Table 1 The blockade of the PI-3K/AKT, ERK and/or JNK signaling pathways results in the reduction in HSC growth, which is partially eliminated by the inhibition of PPARγ activation

	PD′	Changes in viable cells (%)			
		0 μM	5 μM	10 μM	20 μM
Curcumin	−	75.6±5.1			
Control	+	86.1±4.1*			
Non-treatment	−	100			
Control	+	103.7±2.9			
SP	−		91.6±3.6	84.6±1.1	80.9±1.2
600125	+		93.7±1.2	91.3±0.6*	90.5±2.0*
LY	−		94.3±3.2	90.5±1.8	78.5±4.5
294002	+		97.4±10.3	94.5±3.3*	82.8±7.0*
PD	−		96.3±12.0	88.5±1.2	74.1±1.2
98059	+		98.7±9.9	93.7±4.4*	85.2±1.9*
Mixed	−		79.3±3.3	72.2±2.7	62±0.9
Inhibitors	+		84.3±1.5*	77.7±1.4*	66±3.3*

Serum-starved HSC were treated with or without curcumin (20 μM), or LY294002 (LY), or PD98059 (PD), or SP600125 (SP), or a mixture of the three inhibitors at the indicated concentrations in DMEM with FBS (10%) for 24 h in the presence or absence of the PPARγ antagonist PD68235 (PD′) at 20 μM. Cell growth was analyzed by MTS assays. Results were expressed as changes in viable cells (%) (Means±s.d.) (*n*3), compared to the negative control with no treatment. *$P<0.05$ versus cells with the same specific inhibitor at the same concentration.

activation of PPARγ, LY294002, SP600125 or PD98059, like curcumin, reduced the number of viable cells of HSC in a dose-dependent manner. On comparing to each of the individual inhibitor, the mixture of the three inhibitors had a more potent inhibitory effect on HSC growth. For the second group, which had the pretreatment, it is of interest to observe that the inhibition of PPARγ activation by the pretreatment of cells with PD68235 (PD′) partially, but significantly, eliminated the inhibitory effect of each of the inhibitors, as well as the mixture of the three inhibitors (Table 1). Taken together, these results indicated that the blockade of the PI-3K/AKT, JNK and/or ERK signaling pathways resulted in the reduction in HSC growth, which was partially eliminated by inhibition of PPARγ activation. These results collectively suggested that the inhibition of cell growth of HSC by curcumin might result from the blockade of the PI-3K/AKT, ERK and JNK signaling, leading to the induction of gene expression of PPARγ.

The Interruption of the PI-3K/AKT, ERK and JNK Signaling Pathways Induces Apoptosis of Activated HSC, which is Partially Counteracted by Inhibition of PPARγ Activation

Our prior results indicated that PPARγ activation was a prerequisite in the curcumin induction of apoptosis of HSC.[22,23] Additional experiments were performed to evaluate the effect of the interruption of the PI-3K/AKT, ERK and JNK signaling pathways on the curcumin induction of apoptosis of HSC. Serum-starved HSC were similarly divided into two groups, and pretreated with or without the specific PPARγ inhibitor PD68235 (PD′) (20 μM) for 30 min before the addition of curcumin (20 μM), LY294002 (LY), PD98059 (PD) or SP600125 (SP) at the indicated concentrations in the media with FBS (10%) for an additional 24 h. A portion of the cells was used for determination of apoptosis by flow cytometric analyses. Whole-cell extracts from the rest of the cells were prepared for analyses of cyclin D1 (Cln D1), a critical protein in stimulating cell cycle at the stage of G_0/G_1, and the apoptosis relevant proteins Bax and Bcl-2. As shown in Figure 6 by Western blotting analyses, compared with the control with no treatment (the second well in each blot), like curcumin (the first well in each blot), each of the inhibitors caused a dose-dependent reduction in the abundance of cyclin D1. On comparing with the control (the left well in each pair of a kinase inhibitor at the same dose), the pretreatment with the specific PPARγ inhibitor partially eliminated the inhibitory effect of the kinase inhibitor and increased the abundance of cyclin D1 (the right well in each pair). The levels of Bcl-2 and Bax determine the fate of cells, that is, survival or apoptosis. Also as shown in Figure 6, compared with the control with no treatment (the second well in each blot), curcumin, as expected, increased the level of proapoptotic Bax and reduced the abundance of antiapoptotic Bcl-2 (the first well in each blot). Like curcumin, each of

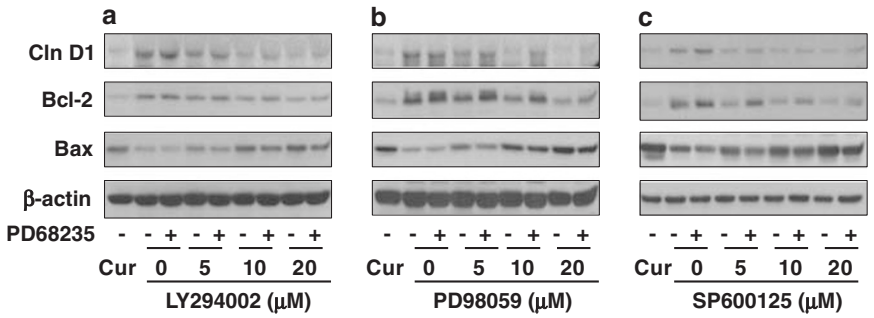

Figure 6 The interruption of PI-3K/AKT, JNK or ERK signaling alters the expression of proteins relevant to cell cycle and apoptosis in activated HSC. Serum-starved HSC were divided into two groups. One group was treated with curcumin (20 μM), or one of the kinase inhibitors at the indicated concentrations, in DMEM with FBS (10%) for 24 h. The other group was pretreated with PD68235, a specific PPARγ inhibitor, at 20 μM for 30 min before the addition of curcumin (20 μM), or one of the inhibitors at the indicated concentrations in the media with FBS (10%) for an additional 24 h. Whole-cell extracts were prepared for Western blotting analyses. The levels of the cell cycle-stimulating protein Cyclin D1, the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax were, respectively, detected. Representatives from three independent experiments were shown. (**a**) Cells were treated with LY294002 with or without the PPARγ antagonist PD68235; (**b**) Cells were treated with PD98059 with or without the PPARγ antagonist PD68235; (**c**) Cells were treated with SP600125 with or without the PPARγ antagonist PD68235.

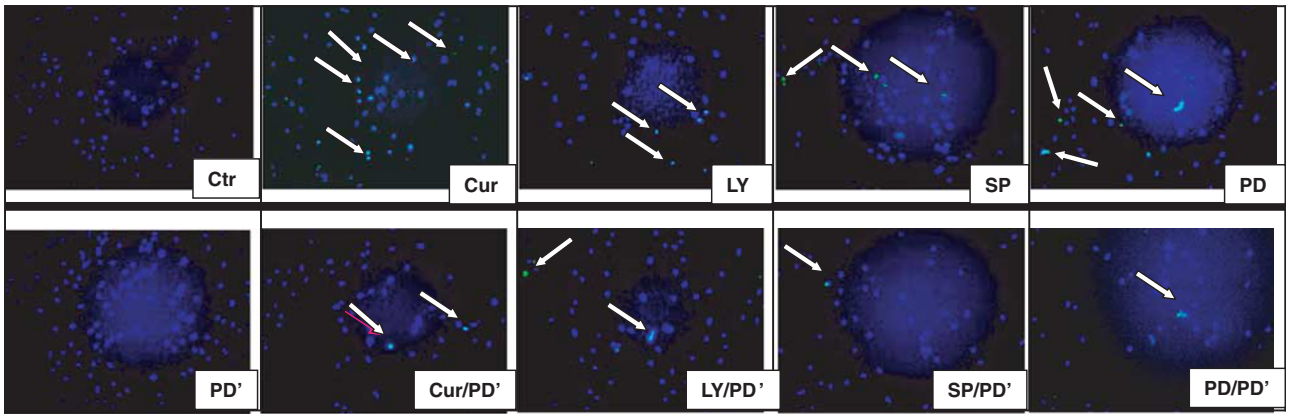

Figure 7 The blockade of the PI-3K/AKT, ERK, and/or JNK signaling pathways results in the induction of apoptosis, which is partially eliminated by the inhibition of PPARγ activation. Serum-starved HSC were treated with or without curcumin (20 μM), or LY294002 (LY) (10 μM), or PD98059 (PD) (10 μM), or SP600125 (SP) (10 μM) for 24 h in the presence or absence of the PPARγ antagonist PD68235 (PD′) at 20 μM. Apoptotic HSC were detected by DeadEnd™ Fluorometric TUNEL System (Promega) and viewed using a fluorescent microscope. Nuclei of apoptotic cells were detected and labeled with green fluorescence indicated by arrows. Nuclei of HSC were stained in blue with DAPI. Representative views ($n = 3$) from each treatment were presented (original × 40).

the inhibitors caused a dose-dependent reduction in the abundance of Bcl-2 and an increase in the level of Bax. Inhibition of PPARγ activation by the pretreatment with PD68235 partially abrogated the ability of the inhibitors to alter the levels of the proteins relevant to apoptosis. The ability of the inhibitors to induce apoptosis was confirmed by TUNEL staining (Figure 7) and by flow cytometric analyses (Table 2). Similarly, this ability was partially eliminated by inhibition of PPARγ activation with PD68235 (PD′). Taken together, these results demonstrated that the blockade of the PI-3K/AKT, ERK and JNK signaling pathways induced apoptosis of activated HSC, which was partially counteracted by inhibition of PPARγ activation.

DISCUSSION

HSC activation is mainly triggered by the release of mitogenic PDGF, EGF and fibrogenic transforming growth factor-β 1 (TGF-β1) from Kupffer cells and activated HSC.[38] The process of HSC activation is coupled with the sequential up-expression of receptors for growth factors, including PDGF-βR, type I & II receptors for TGF-β[39,40] and EGFR.[8] In addition, HSC activation coincides with a dramatic reduction in the abundance PPARγ.[9–11] We previously proposed an antagonistic relationship between the activation of the signaling pathways for these growth factors and the gene expression of PPARγ in HSC, that is, activation of the signaling pathways reduces the activity of PPARγ by suppressing

Table 2 The blockade of PI-3K/AKT, JNK or ERK signaling increases the rate of apoptosis in passaged HSC, which is partially counteracted by the inhibition of PPARγ activation

	PD′	Distributions (%) of apoptotic cells			
		0 μM	5 μM	10 μM	20 μM
Curcumin	−	7.30±0.4			
Control	+	4.6±0.0.3*			
Non-treatment	−	3.67±0.1			
Control	+	3.35±0.1			
SP	−		4.31±0.7	4.88±0.1	6.18±0.7
600125	+		3.93±0.2	4.10±0.2*	4.43±0.3*
LY	−		4.20±0.3	4.98±0.5	6.44±0.3
294002	+		3.86±0.3	4.09±0.5*	4.30±0.4*
PD	−		4.34±0.3	5.05±0.7	6.32±1.2
98059	+		3.99±0.1	4.12±0.4*	4.44±0.1*

Serum-starved HSC were divided into two groups. One group was treated with curcumin (20 μM), or LY294002, or PD98059, or SP600125 at the indicated concentrations, in DMEM with FBS (10%) for 24 h. The other group was pretreated with the specific PPARγ inhibitor PD68235 (PD′) at 20 μM for 30 min prior to the addition of curcumin (20 μM), or one of the inhibitors at the indicated concentrations in the media with FBS (10%) for an additional 24 h. The rate of apoptotic cells was determined by flow cytometric analyses. Distributions (%) of apoptotic cells in the cell cycle in each group were expressed as means±s.d. ($n=3$). *$P<0.05$ versus cells only with the same inhibitor at the same concentration, without the pretreatment with the PPARγ inhibitor.

PPARγ gene expression during HSC activation, whereas activation of PPARγ results in the interruption of the signaling pathways, leading to the inhibition of HSC activation.[24] Our recent results supported the proposal and demonstrated an antagonistic relationship between the activation of TGF-β signaling and the gene expression of PPARγ in HSC.[24] The attention of the current study was paid to the role of PDGF and/or EGF signaling in the curcumin induction of PPARγ gene expression in activated HSC. Results in this report demonstrated that exogenous PDGF or EGF caused a dose-dependent reduction in PPARγ gene expression in passaged HSC (Figure 1). Curcumin interrupted the signaling pathways for PDGF and EGF in activated HSC by reducing the level of tyrosine phosphorylation of PDGF-βR and EGFR and inhibiting the activity of ERK, JNK and PI3/AKT (Figures 2 and 3). The activity of the PI-3K/AKT, ERK or JNK signaling pathway negatively regulated gene expression of PPARγ in activated HSC *in vitro* (Figures 4 and 5). The blockade of PI-3K/AKT, ERK or JNK signaling stimulated PPARγ expression (Figure 5). In addition, the interruption of the PI-3K/AKT, ERK and JNK signaling pathways resulted in the reduction in cell growth and the induction of apoptosis of activated HSC, which were partially eliminated by the inhibition of PPARγ activation (Figures 6 and 7 and Tables 1 and 2). Taken together, our results suggested that the activity of PDGF or EGF signaling might negatively regulate gene expression of PPARγ in activated HSC *in vitro*.

One of the dramatic features during HSC activation is enhanced cell growth, triggered by autocrine/paracrine activation of the signaling for PDGF and EGF.[3–6] The MAPK pathways and the PI-3K/AKT pathway are their major downstream signaling cascades.[41–43] In this study, we hypothesized that the activation of PDGF and EGF signaling pathways might suppress gene expression of PPARγ in HSC. Curcumin might interrupt PDGF and EGF signaling, leading to the relief of the inhibitory effect on PPARγ gene expression in activated HSC. Our results in the present report demonstrated that activation of PDGF and/or EGF signaling suppressed gene expression of PPARγ and reduced the abundance of PPARγ in HSC (Figure 1). The activation of the downstream cascades of PDGF and EGF signaling, including PI-3K/AKT, JNK or ERK, reduced the gene promoter activity of PPARγ (Figure 4). In contrast, the inhibition of the downstream cascades induced gene expression of PPARγ (Figure 5). This observation is supported by prior other studies. It was found that PDGF inhibited PPARγ *trans*-activation activity in HSC transfected with a PPARγ cDNA expression plasmid,[9] and that this inhibitory effect of PDGF was blocked by the inhibition of the MAPK signaling cascade.[9]

We previously demonstrated that curcumin inhibited cell proliferation and induced apoptosis of activated HSC.[22–24] The underlying mechanisms remain largely to be defined. The signaling pathways of PI-3K/AKT, ERK and JNK are critically relevant to cell proliferation and cell survival in many cell types, including in HSC.[44–46] Curcumin reduced the level of tyrosine phosphorylation of PDGF-βR and EGFR and inhibited the activity of ERK, JNK and PI3/AKT

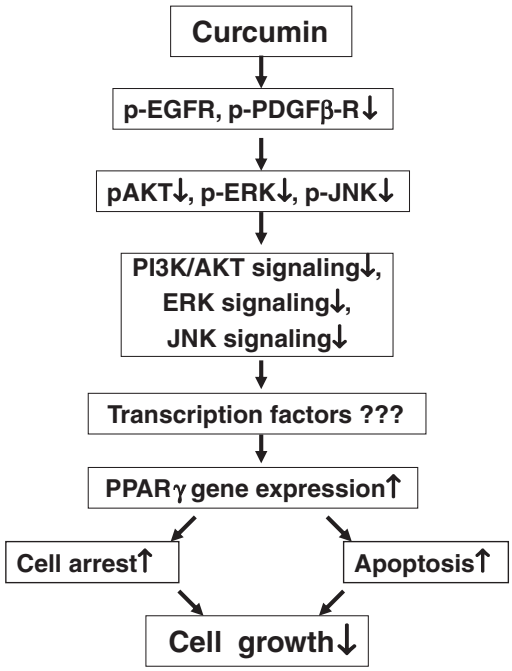

Figure 8 A schematic diagram of the role of the curcumin interruption of PDGF and EGF signaling in the induction of gene expression of PPARγ in activated HSC. Curcumin reduces the level of receptor tyrosine phosphorylation and attenuates the activities of the downstream intermediators AKT, ERK and JNK, leading to the interruption of the signal transduction pathways of PDGF and EGF. These, together with other actions, collectively relieve the inhibitory effects on gene expression of PPARγ, leading to the inhibition of cell growth by inducing cell arrest and apoptosis.

(Figures 2 and 3), leading to the interruption of the mitogenic PDGF and EGF signaling pathways. Our results further demonstrated that the inhibition of PI-3K/AKT, ERK or JNK activity by specific inhibitors dose-dependently reduced cell growth and induced apoptosis of passaged HSC (Figures 6 and 7 and Tables 1 and 2). Moreover, it was of interest to observe that the inhibitory effect was, partially but significantly, abrogated by the pretreatment of cell with PD68235, a special inhibitor of PPARγ activity (Figures 6 and 7 and Tables 1 and 2). These results suggested that the induction of PPARγ gene expression by curcumin and the stimulation of its activity might mediate the effect of the blockade of the PI-3K/AKT, ERK and JNK signaling pathways on the inhibition of cell growth of HSC. Additional experiments using siRNA to specifically block the curcumin induction of PPARγ expression will directly demonstrate the involvement of PPARγ gene expression in the inhibitory process.

Based on our prior and current observations, a model is proposed in Figure 8 to explain the mechanisms of curcumin in the induction of PPARγ gene expression and the inhibition of cell growth, focusing on the involvement of PDGF and EGF signaling in the process. Curcumin interrupts the signaling pathways for PDGF and EGF by reducing the level of receptor tyrosine phosphorylation and suppressing the activity of mitogenic PI-3K/AKT, JNK and ERK, leading to the induction of gene expression of PPARγ and the stimulation of its *trans*-activation activity. The latter mediates the curcumin inhibition of cell growth of activated HSC by inducing cell arrest and apoptosis. In summary, curcumin interrupts PDGF and EGF signaling, which might relieve their inhibitory effects on PPARγ gene expression, leading to the induction of gene expression of PPARγ and the reduction of cell growth of activated HSC *in vitro*. Additional experiments are necessary to clarify downstream transcription factors involved in the regulation of PPARγ gene expression. It bears emphasis that our results and this model do not exclude any other mechanisms in the curcumin induction of PPARγ gene expression and in the inhibition of HSC growth. The results in this report demonstrated that the activity of PDGF and EGF signaling negatively regulated gene expression of PPARγ, which supported our original hypothesis that the interruption of the PDGF and EGF signaling pathways by curcumin might stimulate gene expression of PPARγ in activated HSC *in vitro*. These results provide novel insights into the mechanisms of curcumin in the induction of PPARγ gene expression and in the curcumin inhibition of HSC activation *in vitro*.

ACKNOWLEDGEMENT

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