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Activation of peroxisome proliferator-activated receptor-γ contributes to the inhibitory effects of curcumin on rat hepatic stellate cell growth

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Xu, Jianye, Yumei Fu, and Anping Chen. Activation of peroxisome proliferator-activated receptor-γ contributes to the inhibitory effects of curcumin on rat hepatic stellate cell growth. *Am J Physiol Gastrointest Liver Physiol* 285: G20–G30, 2003. First published March 26, 2003; 10.1152/ajpgi.00474.2002.—Hepatic fibrogenesis occurs as a wound-healing process after many forms of chronic liver injury. Hepatic fibrosis ultimately leads to cirrhosis if not treated effectively. During liver injury, quiescent hepatic stellate cells (HSC), the most relevant cell type, become active and proliferative. Oxidative stress is a major and critical factor for HSC activation. Activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) inhibits the proliferation of nonadipocytes. The level of PPAR-γ is dramatically diminished along with activation of HSC. Curcumin, the yellow pigment in curry, is a potent antioxidant. The aims of this study were to evaluate the effect of curcumin on HSC proliferation and to begin elucidating underlying mechanisms. It was hypothesized that curcumin might inhibit the proliferation of activated HSC by inducing PPAR-γ gene expression and reviving PPAR-γ activation. Our results indicated that curcumin significantly inhibited the proliferation of activated HSC and induced apoptosis in vitro. We demonstrated, for the first time, that curcumin dramatically induced the gene expression of PPAR-γ and activated PPAR-γ in activated HSC. Blocking its *trans*-activating activity by a PPAR-γ antagonist markedly abrogated the effects of curcumin on inhibition of cell proliferation. Our results provide a novel insight into mechanisms underlying the inhibition of activated HSC growth by curcumin. The characteristics of curcumin, including antioxidant potential, reduction of activated HSC growth, and no adverse health effects, make it a potential antifibrotic candidate for prevention and treatment of hepatic fibrosis.

apoptosis; antioxidants; fibrogenesis

HEPATIC FIBROGENESIS OCCURS as a wound-healing process after many forms of chronic liver injury, including virus infection, autoimmune liver diseases, and sustained alcohol abuse (3). Hepatic fibrosis eventually results in cirrhosis if not treated effectively. Hepatic stellate cells (HSC), previously termed fat- or vitamin A-storing cells or Ito cells, are the most relevant cell type for the development of liver fibrosis (17). During liver injury, regardless of etiology, HSC become active and *trans*-differentiate into myofibroblast-like cells characterized by an increase in cell proliferation, loss of vitamin A-storing capability, expression of α-smooth muscle actin (α-SMA), and overproduction of extracellular matrix (ECM). Many researchers, from the therapeutic perspective, have focused their attention on searching for novel agents with inhibitory effects on HSC proliferation and activation to prevent hepatic fibrogenesis.

Although underlying mechanisms remain incompletely understood, it is widely accepted that oxidative stress plays crucial roles in HSC activation during liver injury (17, 31, 48). Oxidative stress is formed by an excessive production of reactive oxygen species, which are generated endogenously by all aerobic cells as byproducts of a number of metabolic reactions (16). Oxidative stress has been implicated in many human diseases such as cancer, cardiovascular diseases, and aging (reviewed by Halliwell; Ref. 22). Studies have shown that oxidative stress stimulates HSC entry into S phase, nuclear factor (NF)-κB activation, and gene expression (31). The antioxidant vitamin E inhibits the activation of HSC (31) and represses iron-induced rat hepatic fibrogenesis (41). The predominant mechanism of antioxidant protective action is to destroy free radicals. The therapeutic efficacy of current well-known antioxidants, including superoxide dismutase, vitamin E, and ascorbic acid, in treatment of human hepatic fibrosis is, however, generally unimpressive (23). Many polyphenolic compounds in plants, including those found in vegetables, fruits, wine, and tea, exhibit antioxidant activities and are beneficial to human health.

The polyphenol compound curcumin is the main yellow pigment of a popular spice, turmeric, and is widely used as a food colorant. Turmeric is the major ingredient in curry. Besides its dietary use, turmeric has been used in Chinese herbal medicine for skin and gut diseases and wound healing. Curcumin is a potent antioxidant (44). It has shown its ability to inhibit lipid peroxidation (42, 47), nitric oxide synthetase activity (4), production of reactive oxygen species (28), protein kinase C activity (32), and NF-κB activity (46). Curcumin has received attention as a promising dietary supplement for cancer prevention (44) and liver protec-

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tion (11). A recent study indicated that dietary administration of curcumin improved both acute and subacute rat liver injury caused by carbon tetrachloride (39). The protective mechanisms of curcumin remain poorly addressed.

The peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors (21). PPAR forms heterodimers with the retinoid X receptor and binds to specific response elements to induce transcription in response to a variety of endogenous and exogenous ligands, including fatty acids, arachidonic acid metabolites, and synthetic drugs, as reviewed by Forman et al. (15). Of the PPAR isoforms, PPAR-γ is the most widely studied (1). Previous studies indicated that expression of PPAR-γ inhibited PDGF-induced proliferation and migration of vascular smooth muscle cells (18). Three recent studies independently demonstrated that the level of PPAR-γ and its *trans*-activating activity were diminished during HSC activation in vitro, whereas NF-κB and activator protein-1 (AP-1) activities were increased (19, 33, 36). PPAR-γ ligands inhibited cell proliferation and collagen-α_1(I) expression in primary HSC (3–4 days) (36). The dramatic reduction in the abundance of PPAR-γ results in a significant decline in response to exogenous PPAR-γ ligands in activated HSC (19, 33, 36). These findings implied a potential therapeutic value of PPAR-γ ligands in treatment of liver fibrosis if the expression of PPAR-γ can be induced in activated HSC.

The aims of this study were to evaluate effects of curcumin on culture-activated HSC growth and to begin exploring the underlying mechanisms. Our results indicated that curcumin significantly inhibited cell proliferation and induced apoptosis of activated HSC in vitro. In addition, we demonstrated, for the first time, that curcumin dramatically induced the expression of PPAR-γ at levels of transcription and translation as well as revived PPAR-γ *trans*-activating activity in activated HSC. Furthermore, activation of PPAR-γ by curcumin resulted in inhibition of transcription factor NF-κB *trans*-activating activity. Blocking PPAR-γ activation by a specific PPAR-γ antagonist caused a marked reduction in inhibition of activated HSC proliferation. Together, our results have indicated that PPAR-γ activation by curcumin plays critical and significant roles in inhibition of activated HSC growth in vitro.

MATERIALS AND METHODS

Isolation and culture of HSC. HSC were isolated from male Sprague-Dawley rats (<200 g) as previously described (7). Cells were cultured in DMEM supplemented with 10% FBS. HSC aged at *passages 4–8* were used for experiments. Curcumin (purity > 94%) was purchased from Sigma (St. Louis, MO). PD-68235 is a specific PPAR-γ antagonist (5), kindly provided by Pfizer (Ann Arbor, MI). 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2) was purchased from BIOMOL Research Labs (Plymouth Meeting, PA).

Lactate dehydrogenase release assays. Lactate dehydrogenase (LDH) assays were performed as recently described (9). In brief, preconfluent HSC were treated with curcumin at the indicated concentrations for 24 h. LDH in conditioned media was determined as medium LDH. LDH in cell lysates was analyzed as cellular LDH. LDH in DMEM with 10% FBS was defined as contamination arising from FBS and subtracted from medium and cellular LDH. LDH activities were determined by an LDH assay kit (Sigma). Results were shown as percentage of total LDH, i.e., medium LDH%/(medium LDH + cellular LDH).

Determination of cell growth. Semiconfluent HSC (5.5×10^4) grown in DMEM containing 10% FBS were treated with curcumin at the indicated concentrations for the indicated times. After cells were washed, cell growth was determined by attached cell numbers counted by a computer-equipped cell counter (Coulter, Miami, FL). Each treatment was given in triplicate. The experiment was repeated at least three times.

[^{3}H]thymidine incorporation assays. The assay was performed as recently described (9). Briefly, semiconfluent HSC (5.5×10^4) grown in DMEM containing 10% FBS were treated with curcumin at the indicated concentrations for 24 h and subsequently pulsed for 4 h with methyl-[^{3}H]thymidine (1 μCi/ml) (Amersham Life Science, Arlington Heights, IL). Whole lysates were mixed with the scintillation fluid Soluscint O (National Diagnostics, Highland Park, NJ) and were counted by a liquid scintillation analyzer. Results were expressed as counts per minute from triplicate experiments.

Bromodeoxyuridine staining. Preconfluent HSC in slide flasks were incubated in DMEM with 10% FBS with or without curcumin at 30 μM for the indicated times. Two hours before cells were harvested, bromodeoxyuridine (BrdU) was added at a final concentration of 25 μg/ml. Cells were fixed and stained by using a BrdU-staining kit from Zymed, following the protocol provided by the manufacturer.

Detection of apoptotic HSC by TUNEL. Preconfluent HSC cultured in DMEM with 10% FBS in slide flasks were treated with or without curcumin (30 μM) for the indicated times. Cells were washed three times with cold PBS before fixation. Apoptotic HSC were detected by the DeadEnd Colorimetric TUNEL System (Promega), following the protocol provided by the manufacturer.

Caspase-3 activity assays. Caspase-3 activities were measured by using a kit purchased from Promega and by following the protocol provided by the manufacturer. Briefly, semiconfluent HSC were treated with curcumin (30 μM) for the indicated times. Cell lysates were incubated with the substrate DEVD-*p*-nitroanilide (pNA) at 37°C for 60–90 min. Results of the reaction were read by a spectrophotometer at 405 nm. The level of caspase enzymatic activity in the cell lysates is directly proportional to the color reaction. A standard calibration curve of pNA was established by a series dilution of pNA solution provided with the kit. Each treatment was performed at least three times.

Western blotting analysis. Whole cell extracts were prepared from preconfluent HSC. SDS-PAGE with 10% resolving gel was used to separate proteins (25 μg/well). Separated proteins were detected by using primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by utilizing chemiluminescence reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Plasmids and transient transfection. The NF-κB reporter plasmid pNF-κB-Luc was purchased from Clontech Laboratories (Palo Alto, CA). The PPAR-γ reporter plasmid pPPRE-TK-Luc contains three copies of the PPAR-γ-response elements from acyl-CoA oxidase gene linked to the herpes virus thymidine kinase promoter (−105/+51) and luciferase vector, which was a gift from Dr. Kevin J. McCarthy (Louisiana

State University Health Sciences Center in Shreveport). Semiconfluent HSC in six-well plastic plates were transiently transfected using the LipofectAMINE reagent (Life Technologies, Grand Island, NY). Each sample (3 μg/well) treatment had three repeats in each experiment. Luciferase assays were performed as previously described (8). Transfection efficiency was determined by cotransfection of a β-galactosidase reporter, pSV-β-gal (0.5 μg/well; Promega). β-Galactosidase activities were measured by a chemiluminescence assay kit (Tropix, Bedford, MA) according to the manufacturer's instructions. Results were combined from three independent experiments.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as previously described (8). The integrity of nuclear extracts was tested by EMSA with a ^{32}P-labeled specificity protein-1 (SP-1) consensus probe, resulting in distinct SP-1 shifts from all extracts (data not shown). The NF-κB probe containing consensus NF-κB binding sites was purchased from Santa Cruz Biotechnology.

RNA isolation and real-time PCR. Total RNA was isolated by TRI-Reagent (Sigma), following the protocol provided by the manufacturer. Real-time PCR was carried out as recently described (9). mRNA fold changes in target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen et al. (45). Primers used in real-time PCR were: collagen-α_1(I) forward 5′-CCT CAA GGG CTC CAA CGA G-3′, reverse 5′-TCA ATC ACT GTC TTG CCC CA-3′; α-SMA forward 5′-CCG ACC GAA TGC AGA AGG A-3′, reverse 5′-ACA GAG TAT TTG CGC TCC GGA-3′; fibronectin forward 5′-TGT CAC CCA CCA CCT TGA-3′, reverse 5′-CTG ATT GTT CTT CAG TGC GA-3′; PPAR-γ forward 5′-ATT CTG GCC CAC CAA CTT CGG-3′, reverse 5′-TGG AAG CCT GAT GCT TTA TCC CCA-3′; bcl-2 forward 5′-ATG GGG TGA ACT GGG GGA TTG-3′, reverse 5′-TTC CGA ATT TGT TTG GGG CAG GTC-3′; GAPDH forward 5′-GGC AAA TTC AAC GGC ACA GT-3′, reverse 5′-AGA TGG TGA TGG GCT TCC C-3′.

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 controls were analyzed by ANOVA with the Dunnett's test for post hoc analysis.

RESULTS

Curcumin causes a dose-dependent inhibition of passaged HSC growth. To evaluate the effect of curcumin on HSC growth, preconfluent HSC grown in DMEM with 10% FBS were treated with curcumin at the indicated concentrations for 24 h. Cell growth was determined by cell numbers. As shown in Fig. 1, compared with control, curcumin caused a significant dose-dependent reduction in cell numbers by 43, 47, 59, and 63% at 20, 30, 40, and 50 μm, respectively. Curcumin toxicity to passaged HSC was carefully studied by examining LDH release and trypan blue exclusion assays. As shown in Table 1, curcumin, compared with control, made no significant difference in LDH release at concentrations up to 100 μM. Trypan blue exclusion assays demonstrated that, compared with no treatment (control), curcumin caused no significant increase in trypan blue-stained cells up to 200 μM (data not shown). On the basis of these observations and a rapid recovery of cell proliferation after withdrawal of curcumin (data not shown), it was concluded that curcumin up to 100 μM was not toxic to cultured HSC. Curcumin at 30 μM was chosen for the following experiments.

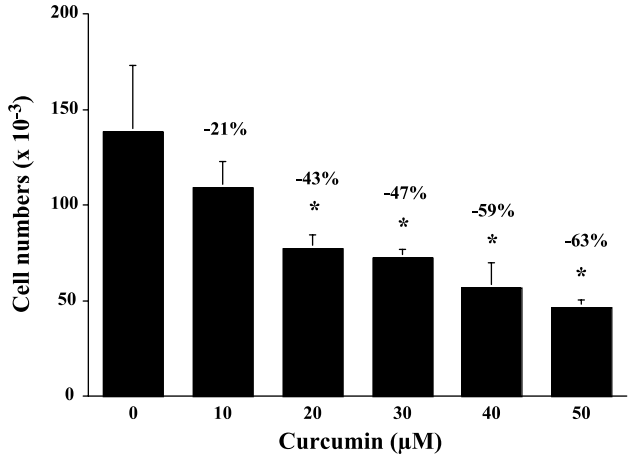

Fig. 1. Curcumin results in a dose-dependent inhibition of passaged hepatic stellate cell (HSC) growth. Preconfluent HSC grown in DMEM with 10% FBS were treated with curcumin at the indicated concentrations for 24 h. After cells were washed, cell growth was determined by attached cell numbers counted by a computer-equipped cell counter. Values are means ± SD of %inhibition caused by curcumin compared with cells with no curcumin (0 μM); $n \geq 6$; *$P < 0.05$ vs. cells with no curcumin treatment.

Curcumin inhibits the proliferation of passaged HSC. To begin elucidating mechanisms underlying the inhibition of cultured HSC growth by curcumin, we hypothesized that this antioxidant might reduce HSC proliferation and/or induce HSC apoptosis. To test this hypothesis, cell proliferation was assessed by analyzing the incorporation of methyl-[^{3}H]thymidine or BrdU into chromosomal DNA. Passaged HSC were treated with or without curcumin for 24 h at the indicated concentrations and pulsed with methyl-[^{3}H]thymidine or BrdU for 4 or 2 h, respectively. As shown in Fig. 2*A*, compared with control (0 μM), curcumin at 30, 50, and 100 μM significantly reduced [^{3}H]thymidine incorporation by 45.7, 50.6, and 52.9%, respectively, suggesting that curcumin, in a dose-dependent manner, inhibited DNA synthesis and cell proliferation. These results were confirmed by BrdU staining of cultured HSC (Fig. 2*B*). Western blotting analyses were carried out to further evaluate the effect of curcumin on the expression of proteins related to the cell cycle. As shown in Fig. 3, curcumin, in a time-dependent manner, markedly reduced the abundance of cell cycle-stimulating proteins, including cyclin D_1, D_2, and E. In addition, this polyphenol enhanced the levels of cell cycle inhibitory proteins, including $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. Together, these results indicated that curcumin inhibited cell proliferation of activated HSC.

Curcumin induces apoptosis in activated HSC. Additional experiments were performed to evaluate the effect of curcumin on HSC survival. Passaged HSC, treated with curcumin, were stained by TUNEL. As shown in Fig. 4*A*, curcumin increased the number of TUNEL-stained cells in a time-dependent manner,

Table 1. *Lactate dehydrogenase release in cultured HSC treated with curcumin*

	Curcumin, μM							
	0	10	20	30	50	100	500	1000
%total LDH	5.29 ± 0.78	4.8 ± 0.36	4.68 ± 0.73	4.36 ± 0.46	4.37 ± 0.76	5.82 ± 0.57	22.8 ± 2.26	21.9 ± 3.12

Values are means ± SD; $n \geq 3$. Lactate dehydrogenase (LDH) release was calculated as medium LDH/(medium LDH + cellular LDH).

suggesting an increase in apoptotic cells. Caspase-3 is an executive enzyme for cell apoptosis. Caspase-3 activity assays demonstrated that curcumin, compared with control, significantly increased the enzyme activity by 6.2-fold after 24 h of treatment (Fig. 4*B*). Real-time PCR further showed that curcumin reduced the mRNA level of Bcl-2, an antiapoptotic protein, by 82% after 24 h of treatment (Fig. 4*C*). Together, these results demonstrated that curcumin induced time-dependent apoptosis in activated HSC.

Curcumin reduces mRNA levels of collagen-α1(I), fibronectin, and α-SMA in passaged HSC. As mentioned earlier, HSC activation is characterized by expression of α-SMA and overproduction of ECM components, including collagen-α1(I) and fibronectin. Further experiments were to evaluate the effect of curcumin on the expression of ECM and α-SMA. Passaged HSC were treated with curcumin (30 μM) for the indicated times. Total RNA was prepared for real-time PCR assays. Endogenous GAPDH was used as an internal control. As shown in Fig. 5, curcumin, in a time-dependent manner, reduced the steady-state levels of mRNA of collagen-α1(I), fibronectin, and α-SMA in passaged HSC.

Curcumin induces the expression of PPAR-γ and activates its trans-activating activity in HSC. Additional studies were focused on elucidating the mechanisms by which the antioxidant curcumin inhibited cell proliferation of activated HSC. Inhibition of cell growth and proliferation by activation of PPAR-γ has been reported in several cell types (20, 30). Recent studies have shown that PPAR-γ is highly expressed in quiescent HSC in normal livers (19, 33, 36). However, its abundance and its *trans*-activating activity are diminished during HSC activation (19, 33, 36). PPAR-γ ligands inhibit HSC proliferation and collagen-α1(I) expression in vitro (36). The antioxidants troglitazone and α-tocopherol induce the expression of PPAR-γ in nonadipose tissues and cell lines (12, 14, 40). These results prompted us to hypothesize that the antioxidant curcumin might induce the expression of PPAR-γ in activated HSC in vitro and that activation of PPAR-γ might contribute to the growth-inhibitory effect of curcumin on activated HSC. To test the hypotheses, initial experiments were designed to determine the capability of curcumin in inducing the expression of PPAR-γ and in activating its *trans*-activating activity in activated HSC in vitro. Passaged HSC were treated with curcumin at 30 μM for the indicated times. Total RNA or protein extracts were prepared from the cells. As demonstrated by real-time PCR in Fig. 6*A*, compared with no treatment (0 h), curcumin significantly enhanced the abundance of PPAR-γ mRNA by 145, 210, and 390% in passaged HSC after the treatment for 8, 16, and 24 h, respectively. Western blotting analyses confirmed the role of curcumin in increasing the protein level of PPAR-γ in passaged HSC (Fig. 6*B*). These results collectively demonstrated that curcumin induced the expression of PPAR-γ in activated HSC.

Further experiments were to verify the effect of curcumin on stimulating the *trans*-activating activity of PPAR-γ. Passaged HSC were transfected with the PPAR-γ reporter plasmid pPPRE-TK-Luc, containing three copies of PPAR-γ response elements inserted into a luciferase reporter vector. After recovery, cells were treated with various concentrations of curcumin or

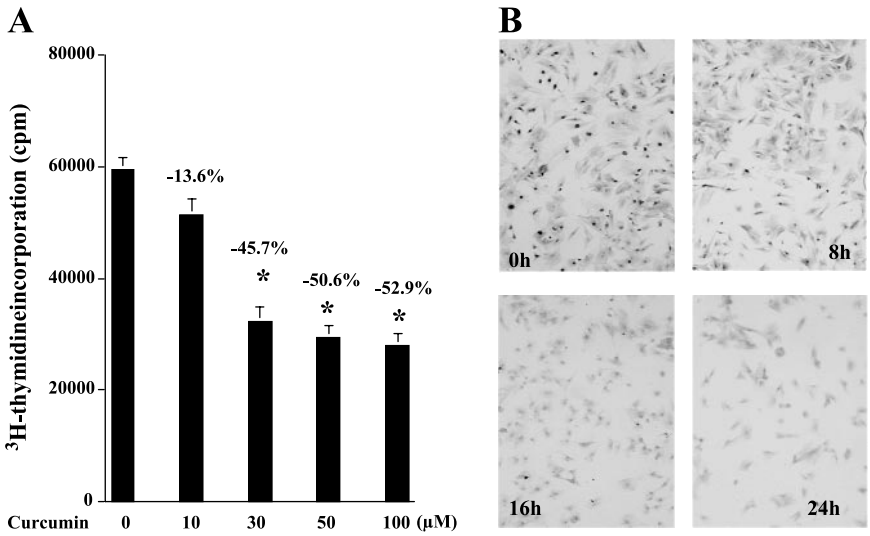

Fig. 2. Curcumin significantly reduces the incorporation of [^{3}H]thymidine and bromodeoxyuridine (BrdU) into cultured HSC cDNA. Passaged HSC were treated with curcumin at the indicated concentrations for 24 h. Methyl-[^{3}H]thymidine (1 μCi/ml) or BrdU (25 μg/ml) was added to the media 4 or 2 h, respectively, before harvesting (see MATERIALS AND METHODS for details). *A*: determination of [^{3}H]thymidine incorporation into cDNA of cultured HSC. Results (means ± SD) were expressed as counts per minute (cpm) from triplicate experiments expressed as %reduction in cpm caused by curcumin compared with cells with no curcumin (0 μM). *$P < 0.05$ vs. cells without curcumin treatment. *B*: representative of BrdU-stained HSC after curcumin treatment for the indicated times. Pictures were taken from random fields of vision (original magnification, ×100).

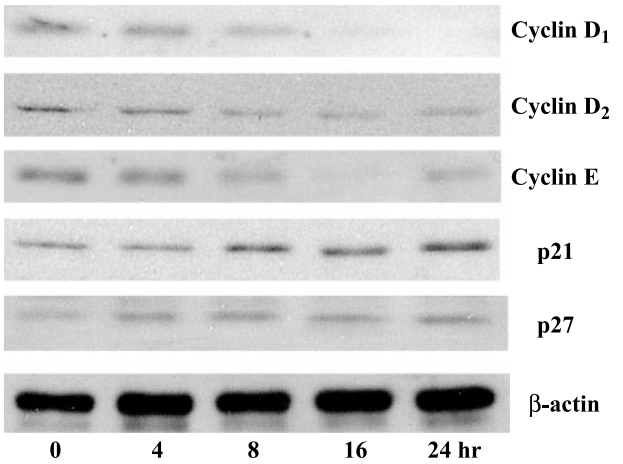

Fig. 3. Curcumin alters the expression of cell cycle-related proteins in activated HSC. Whole cell protein extracts were prepared from preconfluent HSC treated with or without curcumin (30 μM) for the indicated times. SDS-PAGE with 10% resolving gel was used to separate proteins (25 μg/lane). Cell cycle-related proteins were detected by using primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Each result was representative of 3 independent Western blotting analyses. β-Actin was used as an internal control for equal protein loading.

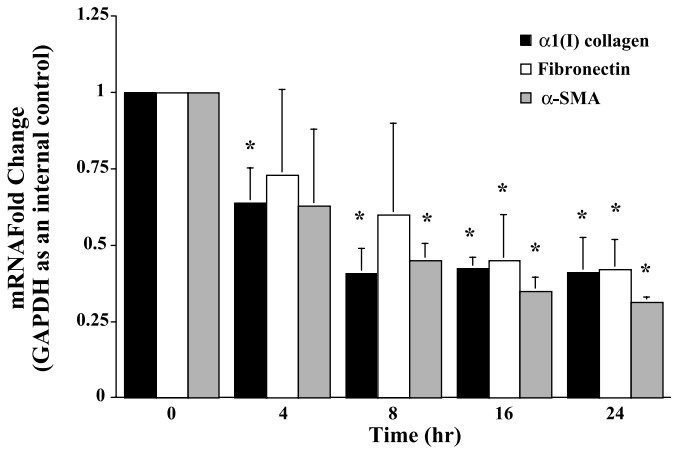

Fig. 5. Curcumin reduces mRNA levels of collagen-α_1(I), fibronectin, and α-smooth muscle actin (α-SMA). Passaged HSC were treated with curcumin at 30 μM for the indicated times. The steady-state mRNA levels of collagen-α_1(I), fibronectin, and α-SMA in cells were determined by real-time PCR. mRNA fold changes were calculated by using GAPDH as an internal control (see MATERIALS AND METHODS for details). Values are means ± SD from 3 independent experiments. *$P < 0.05$ vs. cells without curcumin treatment (0 h).

PGJ_2, a natural PPAR-γ ligand. As shown in Fig. 6*C*, compared with control (0 μM), curcumin at 20, 30, and 50 μM caused dose-dependent, significant increases in luciferase activity by 210, 450, and 510%, respectively. PGJ_2 alone at 5 and 10 μM, however, led to relatively smaller increases in luciferase activity by 150 and 190%, respectively, suggesting that PGJ_2 might only activate endogenous PPAR-γ expressed at a relatively low level in activated HSC. PGJ_2 does not induce PPAR-γ gene expression in activated HSC (data not shown). Further transfection analyses demonstrated that pretreatment of the transfected cells with PD-68235 (20 μM), a specific PPAR-γ antagonist (5), apparently blocked the increase in luciferase activity induced by curcumin (30 μM), whereas PD-68235 itself had no significant effect on luciferase activity (Fig. 6*D*). In addition, compared with cells treated with curcumin or PGJ_2 alone, cells treated with both curcumin and PGJ_2 showed a further significant increase in luciferase activity, which was completely abolished by PD-68235 pretreatment (Fig. 6*D*). Together, these results demonstrated that curcumin induced the expression of PPAR-γ and activated its *trans*-activating activity in activated HSC. It is presumed that PPAR-γ ligands exist in the media with 10% FBS (34).

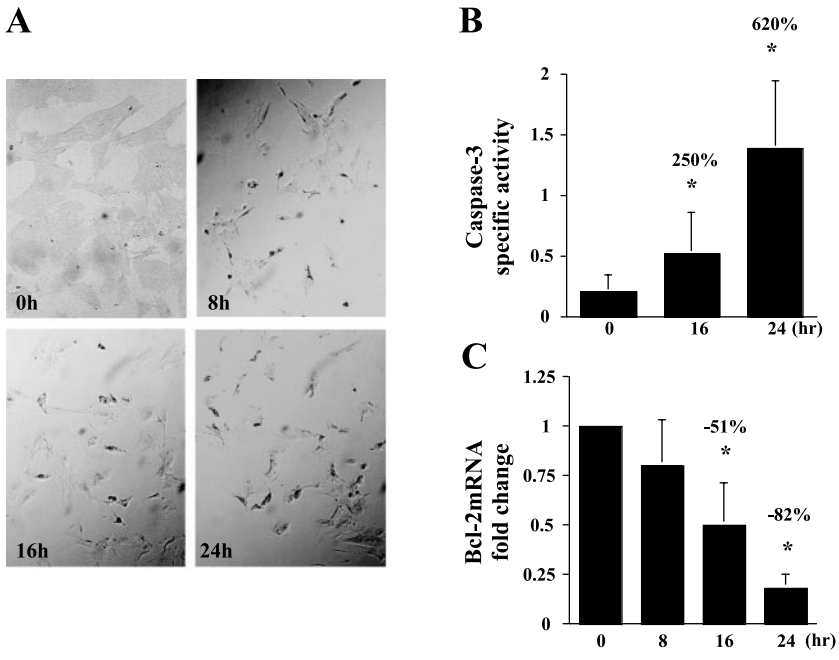

Fig. 4. Curcumin induces HSC apoptosis. Preconfluent HSC were treated with or without curcumin at 30 μM for the indicated times. *A*: representative TUNEL-stained cells (original magnification, ×100). *B*: caspase-3 activities of cells treated with curcumin. Values are %increase in caspase-3 activity caused by curcumin compared with cells with no curcumin (0 h); $n = 6$. *$P < 0.05$ vs. cells without curcumin treatment. *C*: determination of bcl-2 mRNA fold changes by real-time PCR. GAPDH was used as an internal control. Values are %reduction in bcl-2 mRNA fold changes caused by curcumin compared with cells with no curcumin (0 h); $n = 3$. *$P < 0.05$ vs. cells without curcumin treatment.

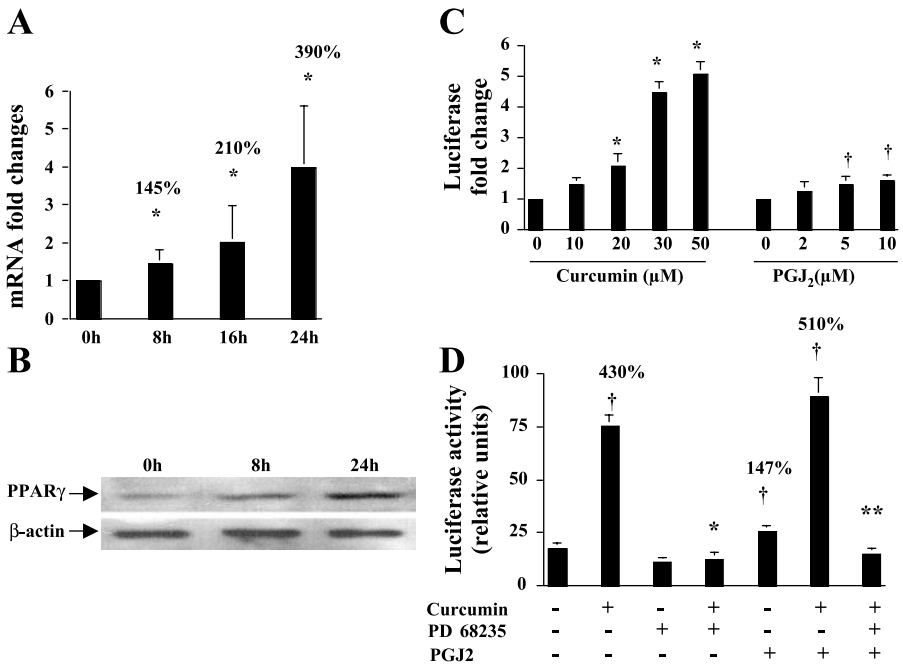

Fig. 6. Curcumin induces the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and activates its *trans*-activating activity in passaged HSC. Passaged HSC were treated with curcumin for indicated times. Samples were prepared for total RNA extraction or protein extraction. *A*: determination of PPAR-γ mRNA fold changes by real-time PCR. GAPDH was used as an internal control. Values are %increase in PPAR-γ mRNA fold changes induced by curcumin compared with cells with no curcumin (0 h); $n = 3$. *$P < 0.05$ vs. cells without curcumin treatment. *B*: representative blot of 3 independent Western blotting analyses of PPAR-γ. β-Actin was used as an internal control for equal protein loading. *C*: semiconfluent HSC were transfected with the PPAR-γ reporter plasmid pPPRE-TK-Luc. Cells were then treated with curcumin or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2) at the indicated concentrations for 36 h. Luciferase activities were expressed as relative units after β-galactosidase normalization; $n = 6$. *$P < 0.05$ vs. cells without curcumin treatment (0 μM); †$P < 0.05$ vs. cells without PGJ_2 treatment (0 μM). *D*: HSC, transfected with pPPRE-TK-Luc, were pretreated with or without PD-68235 (20 μM) for 30 min before the addition of curcumin (30 μM) plus or minus PGJ_2 (5 μM) for an additional 36 h. Luciferase activities were expressed as relative units after β-galactosidase normalization; $n = 6$. †$P < 0.05$ vs. cells with no treatment. *$P < 0.05$ vs. cells treated with curcumin. **$P < 0.05$ vs. cells treated with curcumin and PGJ_2.

Activation of PPAR-γ plays a critical role in inhibition of NF-κB activity by curcumin. Transcription factor NF-κB has been described as a primary regulator and mediator of oxidative stress. Although the causal relationship remains unknown, previous studies demonstrated that activation and survival of HSC were closely associated with the activation of NF-κB (24, 31, 43). It was, therefore, of interest to elucidate the effect of the antioxidant curcumin on NF-κB activity in HSC. Passaged HSC were treated with curcumin at 30 μM for the indicated times. Nuclear extracts were prepared for EMSA using a ^{32}P-labeled probe containing consensus NF-κB binding sites. As shown in Fig. 7*A*, compared with no treatment (*lane 3*), curcumin caused an apparent reduction in the density of the protein-DNA complex in a time-dependent pattern (*lanes 1* and *2*). Fiftyfold excess of the unlabeled probe competitively caused a marked, if not complete, reduction in the binding band (*lane 4*), suggesting the specificity of the protein binding to the probe. Anti-p50 antibodies, but not normal rabbit IgG (data not shown), resulted in a significant supershift and abolished the NF-κB binding band (*lane 5*). This result illustrated that curcumin reduced NF-κB DNA binding activity in passaged HSC. Further experiments were carried out to evaluate effects of curcumin on NF-κB *trans*-activating activity. HSC were transfected with the NF-κB reporter plasmid pNF-κB-Luc and were treated with or without curcumin at indicated concentrations (Fig. 7*B*). Luciferase assays demonstrated that curcumin, in a dose-dependent manner, significantly reduced luciferase activities, indicating that curcumin inhibited NF-κB *trans*-activating activity in cultured HSC. Previous studies have demonstrated an association between activation of PPAR-γ and inhibition of NF-κB (10, 26, 50). It is plausible to evaluate roles of PPAR-γ activated by curcumin in inhibition of NF-κB in activated HSC. As demonstrated in Fig. 7*C*, pretreatment of cells with the specific PPAR-γ antagonist PD-68235 abolished, in a dose-dependent manner, the effect of curcumin (30 μM) on inhibition of NF-κB *trans*-activating activity. PD-68235 itself increased, although not significantly, luciferase activities at both 5 and 20 μM, which might result from blockade of endogenous PPAR-γ activation by the antagonist. Together, these results demonstrated that the antioxidant curcumin inhibited NF-κB activities mediated by activation of PPAR-γ in activated HSC.

Blocking the activation of PPAR-γ abrogates the growth-inhibitory effect of curcumin on HSC. Additional experiments were carried out to examine our hypothesis that inhibition of activated HSC growth by

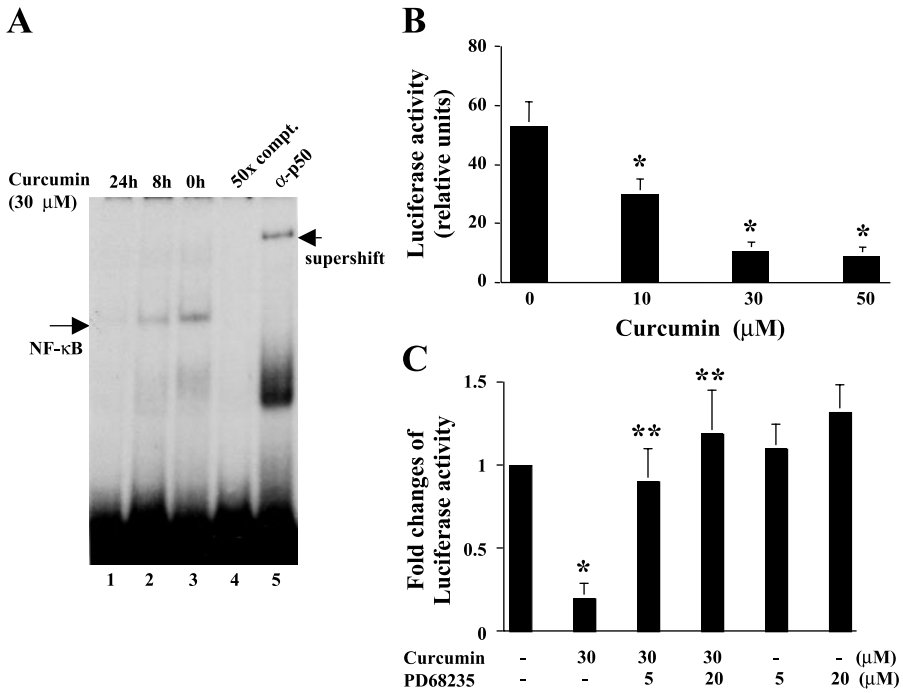

Fig. 7. Activation of PPAR-γ plays a critical role in inhibition of NF-κB *trans*-activating activity by curcumin. *A*: electrophoretic mobility shift assay (EMSA) of the inhibitory effect of curcumin on NF-κB DNA binding activity using a ^{32}P-labeled NF-κB binding probe. Nuclear proteins were prepared from cultured HSC treated with (*lanes 1*, *2*, *4*, and *5*) or without curcumin (30 μM) (*lane 3*) for the indicated times. Fiftyfold excess of the unlabeled probe was used for the competition assay (compt.; *lane 4*). Anti-p50 antibodies (α-p50) were used for the supershift assay (*lane 5*). A representative of 3 independent EMSA is shown. *B*: luciferase assays of the effect of curcumin on NF-κB *trans*-activating activity. After overnight recovery, HSC, transfected with pNF-κB-Luc, were treated with or without curcumin at the indicated concentrations for 36 h. Luciferase activities were expressed as relative units after β-galactosidase normalization; $n \geq 6$. *$P < 0.05$ vs. cells without curcumin (0 μM). *C*: analyses of effects of PPAR-γ activation on NF-κB inhibited by curcumin. HSC, transfected with pNF-κB-Luc, were pretreated with or without PD-68235 (5 or 20 μM) for 30 min before addition of curcumin (30 μM) for an additional 36 h. Luciferase activities were expressed as relative units after β-galactosidase normalization; $n \geq 6$. *$P < 0.05$ vs. cells without curcumin. **$P < 0.05$ vs. cells with 30 μM curcumin only.

curcumin might be mediated by activation of PPAR-γ. Semiconfluent HSC were pretreated with or without the specific PPAR-γ antagonist PD-68235 at the indicated concentrations for 30 min before the addition of curcumin (30 μM) for an additional 24 h. Cells or cell extracts were prepared for determination of cell numbers or Western blotting analyses, respectively. As demonstrated in Fig. 8*A*, curcumin significantly reduced cell numbers, as expected. PD-68235 itself at 10 or 20 μM had no detectable effect on cell numbers. Pretreatment of cells with PD-68235 apparently abrogated the inhibitory effect of curcumin on cell numbers, indicating that blocking the activation of PPAR-γ by PD-68235 abolished the growth-inhibitory effect of cur-

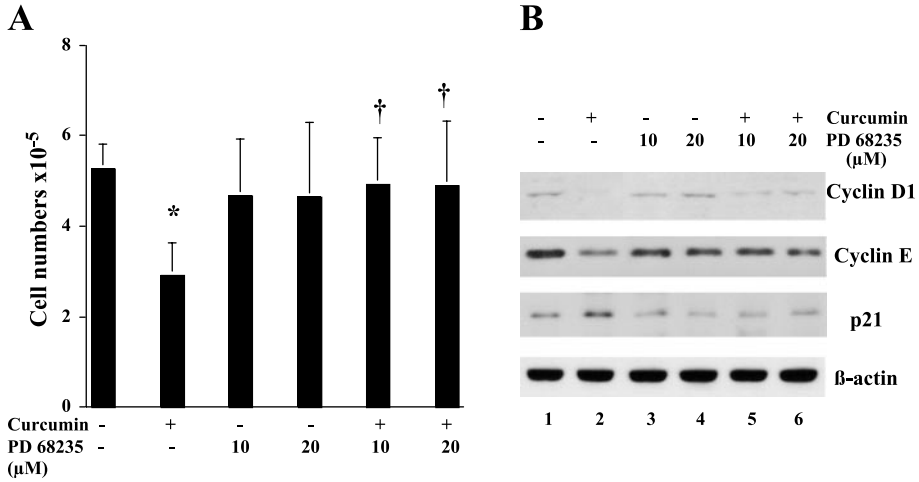

Fig. 8. Blocking the activation of PPAR-γ apparently abrogates the growth-inhibitory effect of curcumin on activated HSC. Preconfluent HSC were pretreated with or without PD-68235 (10 or 20 μM) for 30 min before addition of curcumin (30 μM) for an additional 24 h. Cells and cell extracts were prepared for determination of cell numbers and Western blotting analyses, respectively. *A*: determination of cell numbers; $n = 6$. *$P < 0.05$ vs. cells with no treatment; †$P < 0.05$ vs. cells treated with curcumin only. *B*: representative blot of 3 independent Western blotting analyses. β-Actin was used as an internal control for equal protein loading.

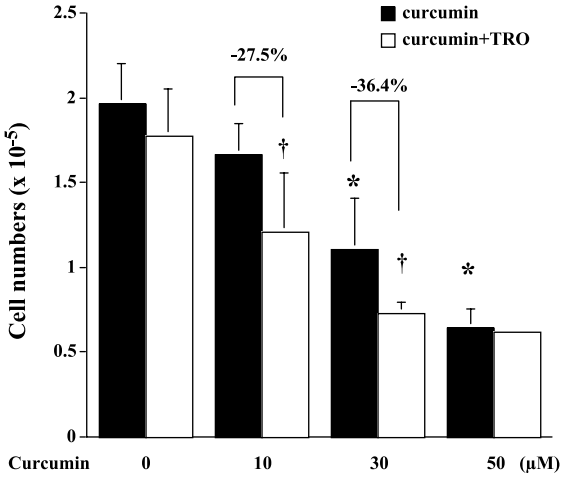

Fig. 9. Troglitazone (TRO) further enhances the inhibitory effect of curcumin on activated HSC growth in vitro. Passaged HSC were pretreated with curcumin at the indicated concentrations for 24 h, with or without the addition of TRO (10 μM) for an additional 24 h. Cell numbers were determined. Values are means ± SD of %reduction in cell numbers by curcumin together with TRO compared with the same group of cells with curcumin only; $n = 6$. *$P < 0.05$ vs. cells with no treatment. †$P < 0.05$ vs. cells treated with the same dose of curcumin.

cumin on activated HSC proliferation. We have demonstrated that curcumin altered the expression of proteins related to the cell cycle (Fig. 3). Further experiments were aimed at the role of PPAR-γ activated by curcumin in the expression of cell cycle-related proteins (Fig. 8*B*). Compared with control (*lane 1*), Western blotting analyses confirmed that curcumin reduced the protein levels of cyclin D_1 and cyclin E (*lane 2*). PD-68235 alone at either 10 or 20 μM had no evident effect on the protein levels (*lanes 3* and *4*). However, PD-68235 pretreatment dramatically abolished the role of curcumin in reducing the protein levels of cyclin D_1 and cyclin E (*lanes 5* and *6*). Similarly, PD-68235 abrogated the role of curcumin in increasing the protein level of $p21^{WAF1/Cip1}$ (*lanes 5* and *6*), a cell cycle-inhibitory protein. These results implied that PPAR-γ activation might be involved in the inhibitory effect of curcumin on cell growth by alteration of levels of proteins involved in cell cycles.

Troglitazone further enhances the curcumin effect on inhibition of activated HSC. We have observed that, compared with either curcumin or PGJ_2 alone, the combination of curcumin and PGJ_2 resulted in a further increase in luciferase activity in HSC transfected with pPPRE-TK-Luc (Fig. 6*D*). We assumed that the PPAR-γ ligand might take advantage of the increase in the abundance of PPAR-γ induced by curcumin and further stimulate the receptor *trans*-activating activity. To study this assumption, passaged HSC were pretreated with curcumin at the indicated concentrations for 24 h to increase the abundance of PPAR-γ. Cells were subsequently treated with or without troglitazone (10 μM), a synthetic PPAR-γ ligand, for an additional 24 h. As shown in Fig. 9, without curcumin pretreatment, troglitazone itself did not significantly reduce cell numbers. In contrast, with curcumin pretreatment at 10 or 30 μM, in addition to the inhibition by curcumin, troglitazone caused a further significant reduction in cell numbers by 27.5 or 36.4%, respectively. MTS assay (Promega), a colorimetric method for determining the number of viable cells, obtained a similar result. These results demonstrated that troglitazone further enhanced the curcumin effect on inhibition of activated HSC and implied that troglitazone might take advantage of the increase in the level of PPAR-γ induced by curcumin to further inhibit the proliferation of activated HSC.

DISCUSSION

In this study, we demonstrated that the antioxidant curcumin significantly inhibited cell growth and suppressed the expression of collagen-α_1(I), fibronectin, and α-SMA genes in passaged HSC. In addition, curcumin induced the expression of PPAR-γ and revived its *trans*-activating activity in activated HSC in vitro, which was found to be mainly responsible for the effects of the antioxidant on inhibition of HSC proliferation and cell growth.

Previous studies have suggested that D-type cyclins might play critical roles in cell cycle progression, especially at the early G_0/G_1 phase (25, 38). Inhibition of cyclin D_1 expression by microinjection of anti-cyclin D_1 antibodies or antisense cyclin D_1 cDNA prevented cells from entering S phase (2). In this study, we observed that curcumin significantly altered the expression of proteins related to the cell cycle in activated HSC. This antioxidant markedly reduced the abundance of cell cycle-stimulating proteins, including cyclin D_1, D_2, and E. In addition, this polyphenol increased the protein levels of cell cycle-inhibitory proteins, including $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, in passaged HSC. A recent study demonstrated that cyclin D_1, D_2, and E played a key role in the transition of the HSC cell cycle from G_1 to S phase (29). Interestingly, blockade of PPAR-γ activation by the antagonist PD-68235 dramatically, if not completely, abrogated the ability of curcumin to alter the expression of cell cycle-related proteins, suggesting that alteration of the expression of cell cycle-related proteins by curcumin might be mediated by PPAR-γ. This result was consistent with a previous observation that PPAR-γ activated by either natural (PGJ_2 and PGD_2) or synthetic ligands (BRL-49653 and troglitazone) selectively inhibited the expression of cyclin D_1 gene mediated by AP-1 (49).

We had previously demonstrated that curcumin blocked JNK activation and inhibited AP-1 activity in passaged HSC (7). We recently observed that another antioxidant (−)-epigallocatechin-3-gallate (EGCG), a major and active component in green tea extracts, also inhibited passaged HSC proliferation and altered the expression of proteins related to the cell cycle (9). Further experiments are necessary to elucidate the mechanisms by which antioxidants regulate the expression of cell cycle-related proteins.

The reduction of levels of PPAR-γ is coupled with the activation of HSC (19, 33, 36), implying a role of PPAR-γ in inhibiting the activation of HSC. In this study, we demonstrated, for the first time, that the antioxidant curcumin induced the expression of PPAR-γ and revived the activation of its *trans*-activating activity in activated HSC in vitro. Induction of PPAR-γ expression is not unique to curcumin. Recent studies showed a unique capability of troglitazone among thiazolidinediones of inducing the expression of PPAR-γ in nonadipose tissues and cell lines (12, 40). The unique antioxidant α-tocopherol moiety in the chemical structure of troglitazone was assumed to be responsible for it (13, 27). Further studies demonstrated that α-tocopherol was also able to induce PPAR-γ expression (14). We recently observed that the antioxidant EGCG in green tea extracts also induced the expression of PPAR-γ in activated HSC (Chen and Zhang, unpublished observations). The mechanisms by which antioxidants induce PPAR-γ gene expression in activated HSC remain poorly understood.

Activation of PPAR-γ by curcumin makes a significant contribution to the inhibitory effect of the antioxidant on activated HSC growth. Blocking PPAR-γ activation by PD-68235 significantly, if not completely, abolished the inhibitory effects of curcumin on cell growth. PPAR-γ has shown its ability to inhibit cell growth and to regulate gene expression in several cell types, including HSC (19, 20, 30, 33, 36). The PPAR-γ ligands troglitazone and PGJ_2 significantly decreased PDGF-induced proliferation in activated human HSC and inhibited α-SMA expression during HSC activation (19). Our preliminary results suggested that activation of PPAR-γ might be involved in inhibition of the expression of the α-SMA gene, but not the collagen-α_1(I) and fibronectin genes, by curcumin in activated HSC (Xu and Chen, unpublished data). It is presumed that PPAR-γ ligands existing in media with 10% FBS initiate the activation of PPAR-γ induced by curcumin in activated HSC. This assumption is supported by a recent observation that platelet-derived lysophosphatidic acid in serum is a transcellular PPAR-γ agonist (34). The effect of curcumin on production of endogenous PPAR-γ ligand(s) is completely unknown.

NF-κB has been described as a primary regulator and mediator of oxidative stress. It has been implicated in cell proliferation, cell cycle regulation, and apoptosis (35). Although the causal relationship remains unknown, previous studies demonstrated that activation and survival of HSC were closely associated with activation of NF-κB (24, 31, 43). It was suggested that inhibition of NF-κB activation might be a potential strategy for prevention and/or treatment of hepatic fibrogenesis (24). In the present studies, we demonstrated that the antioxidant curcumin reduced NF-κB activity in cultured HSC. Previous studies have shown that activation of PPAR-γ resulted in inhibition of NF-κB activity (10, 26, 50). Experiments in this study demonstrated that the PPAR-γ antagonist PD-68235 abrogated the inhibitory effect of curcumin on NF-κB *trans*-activating activity. These results suggested, for the first time, that the inhibitory effect of curcumin on NF-κB activity in activated HSC might be mediated by PPAR-γ activation, which provided a novel insight into the mechanism of the inhibitory effects of curcumin on NF-κB activity. Further studies are necessary to evaluate whether it is unique in activated HSC. It remains incompletely understood how PPAR-γ activation could result in inhibition of NF-κB. It was previously reported that PPAR-γ agonists, including Wy-14643, clofibrate, carbaprostacyclin, and ciglitazone, inhibited NF-κB activity by prevention of IκB from phosphorylation and subsequent degradation in cytokine-stimulated mesangial cells (6). A recent study showed that overexpression of the coactivator p300 restored NF-κB *trans*-activating activity suppressed by a PPAR-γ ligand (37), implying that PPAR-γ might interfere with NF-κB *trans*-activating activity via coactivator competition. Additional studies are necessary to elucidate the causal relationship among NF-κB activities, PPAR-γ activation, and the expression of genes regulated by curcumin.

In summary, our results demonstrated that the antioxidant curcumin inhibited the growth of passaged HSC by reducing cell proliferation and inducing apoptosis. In addition, the polyphenol suppressed the activation of passaged HSC demonstrated by repressing the activity of NF-κB and reducing mRNA levels of collagen-α_1(I) and fibronectin, as well as α-SMA. Furthermore, we reported, for the first time, that curcumin dramatically induced the expression of PPAR-γ and revived its *trans*-activating activity in activated HSC in vitro. Activation of PPAR-γ contributed to the inhibitory effect of curcumin on activated HSC proliferation. It should be emphasized that the results in this study were generated from cultured HSC and that they might not necessarily and comprehensively reflect facts in quiescent HSC in vivo. Further experiments, beyond the scope of this study, are required to elucidate the underlying mechanisms of PPAR-γ in inhibition of HSC proliferation. Our results in this study provided a novel insight into mechanisms of inhibition of activated HSC proliferation by antioxidants, including curcumin. The characteristics of curcumin, including antioxidant potential, inhibition of activated HSC proliferation, induction of apoptosis, activation of PPAR-γ, as well as the long history of dietary consumption of curry without adverse health effects, make it a potential antifibrotic candidate for treatment and prevention of hepatic fibrogenesis.

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