Curcumin suppresses the expression of extracellular matrix genes in activated hepatic stellate cells by inhibiting gene expression of connective tissue growth factor

Zheng, Shizhong, and Anping Chen. Curcumin suppresses the expression of extracellular matrix genes in activated hepatic stellate cells by inhibiting gene expression of connective tissue growth factor. Am J Physiol Gastrointest Liver Physiol 290: G883–G893, 2006. First published November 23, 2005; doi:10.1152/ajpgi.00450.2005.—Upon liver injury, quiescent hepatic stellate cells (HSCs), the most relevant cell type for hepatic fibrogenesis, become active and overproduce extracellular matrix (ECM). Connective tissue growth factor (CTGF) promotes ECM production. Overexpression of CTGF during hepatic fibrogenesis is induced by transforming growth factor (TGF)-β. We recently demonstrated that curcumin reduced cell growth and inhibited ECM gene expression in activated HSCs. Curcumin induced gene expression of peroxisome proliferator-activated receptor (PPAR)-γ and stimulated its activity in activated HSCs, which was required for curcumin to suppress ECM gene expression, including α(I)-collagen. The underlying mechanisms remain largely unknown. The aim of this study was to elucidate the mechanisms by which curcumin suppresses α(I)-collagen gene expression in activated HSCs. We hypothesize that inhibition of α(I)-collagen gene expression in HSCs by curcumin is mediated by suppressing CTGF gene expression through attenuating oxidative stress and interrupting TGF-β signaling. The present report demonstrated that curcumin significantly reduced the abundance of CTGF in passaged HSCs and suppressed its gene expression. Exogenous CTGF dose dependently abrogated the inhibitory effect of curcumin. Activation of PPAR-γ by curcumin resulted in the interruption of TGF-β signaling by suppressing gene expression of TGF-β receptors, leading to inhibition of CTGF gene expression. The phytochemical showed its potent antioxidant property by significantly increasing the level of total glutathione (GSH) and the ratio of GSH to GSSG in activated HSCs. De novo synthesis of cellular GSH was a prerequisite for curcumin to interrupt TGF-β signaling and inhibited gene expression of CTGF and α(I)-collagen in activated HSCs. Taken together, our results demonstrate that inhibition of α(I)-collagen gene expression by curcumin in activated HSCs results from suppression of CTGF gene expression through increasing cellular GSH contents and interruption of TGF-β signaling. These results provide novel insights into the mechanisms underlying inhibition of HSC activation by curcumin.

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depending on sequences present in the 5’-flanking region of the CTGF gene promoter (26). CTGF is transcriptionally regulated by TGF-β and mediates some of the ECM-inducing properties previously attributed to TGF-β (7, 22, 32). The strong correlation between CTGF and TGF-β, as well as the degree of fibrosis, is observed in cirrhotic liver tissue samples (42).

Oxidative stress is a major player in inducing HSC activation and hepatic fibrogenesis, regardless of etiology (19, 21, 24, 33, 51). Oxidative stress is a deleterious imbalance between the production and removal of reactive radicals, including reactive oxygen species (ROS). ROS, are generated from aerobic cells by various mechanisms, including inflammation, aerobic metabolism, and exposure to ionizing radiation (20, 28, 57). Responses of mammalian cells to oxidative stress occur through several antioxidant systems, including enzymes and nonenzymatic molecules. Among them, glutathione (GSH) is the most abundant thiol antioxidant (54). It reacts directly with ROS or functions as a cofactor of antioxidant enzymes (54). GSH is converted to its oxidized form, GSSG, leading to conversion of H₂O₂ and lipid peroxides to water and lipid alcohols, respectively. The GSH-to-GSSG ratio is regarded as a sensitive indicator of oxidant stress in cells (27). Although the antioxidant vitamin E inhibits HSC activation (33) and hepatic fibrogenesis (43), currently well-known antioxidants in protection of the liver from hepatic fibrosis are generally unimpressive (28). The antioxidant capability of curcumin is far greater than that of vitamin E and/or C (48), due to both the innate molecular structure and its ability to affect enzyme systems involved in the defense against oxidative stress (3, 18). These unique features might allow curcumin to succeed where other antioxidants have failed in inhibition of hepatic fibrogenesis.

The aim of this study was to elucidate the mechanisms by which curcumin suppresses ECM gene expression in activated HSCs. We hypothesized that inhibition of ECM gene expression in HSCs by curcumin might be mediated by suppressing CTGF gene expression through attenuating oxidative stress and interrupting TGF-β signaling. Results from this report support our hypothesis and provide novel insights into the mechanisms underlying the curcumin inhibition of HSC activation.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences center (no. P-05-047).

Isolation and culture of HSCs. HSCs were isolated from male Sprague-Dawley rats (~200–250 g) as previously described (11). Cells were cultured in DMEM supplemented with 10% FBS. HSCs aged at passages 4–8 were used for experiments. All of the experiments were performed in media with 10% FBS unless specific indication. Curcumin (purity >94%) was purchased from Sigma (St. Louis, MO). PD-68235 is a specific PPAR-γ agonist (10) and was kindly provided by Pfizer (Ann Arbor, MI). Active TGF-β1 and CTGF were purchased from Cell Sciences (Canton, MA).

Western blot analyses. Whole cell extracts were prepared from preconfluent passaged HSCs. 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 (Kirkegarrd & Perry Laboratories, Gaithersburg, MD). After normalization with β-actin, compared with no treatment, the effect of curcumin on reducing the level of CTGF was determined and expressed as a mean value (n = 3) using Quantity One 4.4.1 (Bio-Rad).

Results and transient transfection assays. CTGF promoter luciferase (Luc) reporter plasmid pCTGF-Luc, a gift from Dr. Yuqing E. Chen (Cardiovascular Research Institute, Morehouse School of Medicine, Atlanta, GA), contains a fragment of the CTGF gene promoter (~2,000 bp nucleotides) subcloned into the Luc reporter plasmid pGL3 (22). PPAR-γ expression plasmid pPPARγcDNA, containing PPAR-γ cDNA, was a gift from Dr. Reed Graves (Department of Medicine, University of Chicago, Chicago, IL). The plasmid p3TP-Lux is a TGF-β-inducible Luc reporter containing the plasmogen activator inhibitor-1 gene promoter and was kindly provided by Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center). The Luc reporter plasmid TβRII-Luc (pES1.0) was a gift from Dr. Michael Centrella (Yale University) and contains 965 bp of the gene promoter of TGF-β receptor type 1 (TβRII) (30). Luc reporter plasmid pTβRII-Luc was generously provided by Dr. Seong-Jin Kim [Laboratory of Chemical Prevention, National Cancer Institute (NCI), Bethesda, MD]. pTβRII contains 1,670 bp of the gene promoter of TGF-β receptor type 2 (TβRII) (4). cDNA expression plasmid pΔTβRII-LucDNA was a gift from Dr. Robert J. Lechleider (NCI) and contains cDNA encoding the dominant-negative form of TβRII (17). Semi-confluent HSCs in six-well cell culture plates were transiently transfected using LipofectAMINE reagent (Life Technologies, Grand Island, NY). Each sample (3 μg of DNA/well) was triplicate in every experiment. Luc assays were performed as previously described (12).

Transfection efficiency was determined by cotransfection of a β-galactosidase reporter, pSV-β-gal (0.5 μg/well; Promega). β-Galactosidase activity was measured by a chemiluminescence assay kit (Tropix, Bedford, MA) according to the manufacturer’s instructions. Results were combined from three independent experiments.

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 previously described (14). cDNA fold changes of target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen et al. (47). The CTGF primers used in real-time PCR were as follows: forward 5’-TGT GTG ATG AGC CCA AGG AC-3’ and reverse 5’-AGT TGG CTC GCA TCA TAG TTG-3’. Other primers used were previously described (56).

GSH assays. The levels of GSH and GSSG were determined using enzyme immune assay kit GSH-400 (Cayman Chemical, Ann Arbor, MI) following the protocol provided by the manufacturer.

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

RESULTS

Curcumin dose dependently suppresses gene expression of CTGF in activated HSCs. All of the following experiments were performed in media with 10% FBS unless specifically indicated. To evaluate the effect of curcumin on gene expression of CTGF in activated HSCs, passaged HSCs were treated with curcumin at the indicated concentrations for 24 h. Total RNA or whole cell protein extracts were prepared from these cells for real-time PCR or Western blot analyses, respectively.

It was found that curcumin caused a dose-dependent reduction in the steady-state level of CTGF mRNA (Fig. 1A) and in the abundance of CTGF protein (Fig. 1B). Curcumin at 20 μM significantly reduced the levels of CTGF mRNA (Fig. 1A) and protein (Fig. 1B) by ~45% and ~60%, respectively. Similarly,
Curcumin caused a dose-dependent reduction in the concentration of secreted CTGF in the conditioned media (data not shown). To further evaluate the effect of curcumin on the regulation of CTGF gene expression, HSCs were transiently transfected with the CTGF promoter Luc reporter plasmid.
pCTGF-Luc, which contains a fragment of the CTGF gene promoter (~2,000 bp nucleotides) subcloned into the Luc reporter plasmid pGL3 (22). Cells were then treated with curcumin at the indicated concentrations. As shown in Fig. 1C by Luc assays, curcumin reduced Luc activity in a dose-dependent manner, suggesting that curcumin reduced the promoter activity of the CTGF gene in activated HSCs.

We (55, 56) recently demonstrated that curcumin induced gene expression and activation of PPAR-γ in passaged HSCs, which was a necessity for curcumin to reduce HSC growth and suppress ECM gene expression. To determine the role of PPAR-γ activation in the inhibitory effect of curcumin on gene expression of CTGF, cells were pretreated with the PPAR-γ-specific antagonist PD-68235 (10) for 30 min before the addition of curcumin. Inhibition of PPAR-γ activation by PD-68235 resulted in partial abrogation of the inhibitory effect of curcumin on the promoter activity of the CTGF gene (Fig. 1C) and on the levels of CTGF mRNA (Fig. 1A) and protein (Fig. 1B) in passaged HSCs. Taken together, these results demonstrated that curcumin dose dependently suppressed gene expression of CTGF in activated HSCs in vitro, which required, at least in part, activation of PPAR-γ.

**Activation of PPAR-γ results in inhibition of CTGF gene expression in activated HSCs.** Although the level of PPAR-γ is dramatically reduced in activated HSCs, it still responds to its agonists, leading to inhibition of HSC activation and suppression of collagen gene expression in vitro and in vivo (23, 34, 37). We (55) have shown that curcumin induces gene expression of PPAR-γ and stimulates its trans-activation activity in activated HSCs in vitro. The results shown in Fig. 1 suggested that PPAR-γ activation might be required for curcumin to inhibit CTGF gene expression in activated HSCs. Additional experiments were conducted to verify the role of PPAR-γ activation in inhibition of CTGF gene expression. Passaged HSCs were treated with 15-deoxy-Δ12,14-prostaglandin J₂ (PGJ₂), a natural PPAR-γ ligand, at various concentrations for 24 h. As shown in Fig. 2, A and B, activation of endogenous PPAR-γ by PGJ₂ dose dependently suppressed gene expression of CTGF at both levels of transcription and translation. To further confirm the role of PPAR-γ activation in inhibition of CTGF gene expression, passaged HSCs were cotransfected with the CTGF promoter Luc reporter plasmid pCTGF-Luc and the cDNA expression plasmid pPPARγcDNA, which contains PPAR-γ cDNA in a cytomegalovirus-driven expression vector. Prior experiments have shown that forced expression of PPAR-γ cDNA results in an increase in the trans-activation activity of PPAR-γ in HSCs (56). PPAR-γ agonists are presumed to exist in the media with 10% FBS (29, 36, 56). As shown in Fig. 2C by Luc assays, forced expression of exogenous PPAR-γ led to a dose-dependent reduction in Luc activity in these cells, indicating that the activation of PPAR-γ inhibited the promoter activity of the CTGF gene. In summary, these results collectively demonstrated that activation of PPAR-γ, regardless of the endogenous or exogenous receptor, resulted in mimicking the role of curcumin inhibition of CTGF gene expression in activated HSCs.

**Exogenous CTGF dose dependently abrogates the inhibitory effect of curcumin on gene expression of α(I)-collagen in activated HSCs.** We hypothesized that inhibition of the expression of ECM genes, including α(I)-collagen, by curcumin was at least partially mediated by suppression of CTGF gene expression in activated HSCs. To test the hypothesis, HSC were treated with curcumin (20 μM) in the presence or absence of exogenous CTGF at the indicated concentrations for 24 h. Total RNA and whole cell extracts were prepared from these cells. Real-time PCR assays (Fig. 3A) and Western blot analyses (Fig. 3B) demonstrated that curcumin significantly reduced, as expected, the abundance of α(I)-procollagen at levels of translation and transcription. It was of interest to observe that coupled with the increase in the dose of exogenous CTGF, the inhibitory effect of curcumin was gradually diminished. These results collectively demonstrated that exogenous CTGF dose dependently eliminated the inhibitory effect of curcumin, indicating the role of CTGF in mediating the physiochemical inhibition of gene expression of α(I)-collagen in activated HSCs.

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**Fig. 3.** Addition of exogenous CTGF dose dependently eliminates the inhibitory effect of Cur on gene expression of α(I)-procollagen in activated HSCs in vitro. HSCs were treated with Cur (20 μM) in the presence or absence of exogenous CTGF at the indicated concentrations for 24 h. Total RNA or whole cell extracts were prepared from these cells for analyses of α(I)-collagen gene expression by real-time PCR assays (A; n = 3) or Western blot analyses (B; n = 3), respectively. mRNA fold changes were calculated by using GAPDH as an invariant control in real-time PCR. *P < 0.05 vs. cells with no treatment; **P < 0.05 vs. cells treated with Cur but without CTGF. β-Actin was an internal control for equal loading in Western blot analyses. Representative images from 3 independent experiments are shown. The numbers beneath α(I)-procollagen are means (n = 3) of the amount of α(I)-procollagen after normalization with β-actin and comparison with the control.
Fig. 4. Cur interrupts transforming growth factor (TGF-β) signaling, leading to inhibition of CTGF gene expression. A: passaged HSCs were transfected with p3TP-Lux. Cells were treated with Cur at the indicated concentrations for 24 h. Luc activity is expressed as relative units after β-galactosidase normalization (n = 6). *P < 0.05 vs. cells with no treatment. B: HSCs in 6-well cell culture plates were cotransfected with pCTGF-Luc and pdn-Tβ-RII at the indicated concentrations. pdn-Tβ-RII contains 1,670 bp of the gene promoter of TGF-β/H9252. A total of 4.5 μg of plasmid DNA were used in each well for transfection, including 2 μg of pCTGF-Luc, 0.5 μg of pSV-β-gal, pdn-Tβ-RII at the indicated doses, and the empty vector pcDNA. The amount of pdn-Tβ-RII plus pcDNA was equalized to 2 μg. Cells were treated with or without Cur at 20 μM for 24 h. Luc activity is expressed as relative units after β-galactosidase normalization (n = 6). Values are expressed as means ± SD. *P < 0.05 vs. cells cotransfected with no pdn-Tβ-RII.

**Curcumin interrupts TGF-β signaling, resulting in inhibition of CTGF gene expression in passaged HSCs.** Overproduction of CTGF in a variety of fibrotic disorders is presumably secondary to the activation and production of TGF-β (25). Gene expression of CTGF is primarily regulated by TGF-β in HSCs (41, 44). We, therefore, hypothesized that inhibition of CTGF gene expression by curcumin resulted from interruption of the TGF-β signal transduction pathway in activated HSCs. To study the hypothesis, HSCs were transfected with the TGF-β-inducible Luc reporter plasmid p3TP-Lux. Cells were treated with curcumin at the indicated concentrations for 24 h. The Luc assays shown in Fig. 4A demonstrated that the curcumin treatment of these cells caused a dose-dependent reduction in Luc activity, indicating that curcumin interrupted the TGF-β signaling pathway. To verify the role of the blockade of TGF-β-signaling in inhibition of CTGF gene expression, passaged HSCs were cotransfected with the CTGF promoter reporter plasmid pCTGF-Luc and the plasmid pdn-Tβ-RII at the indicated concentrations. pdn-Tβ-RII contains the fragment of cDNA encoding the dominant-negative form of TGF-β receptor type 2 (Tβ-RII). A total of 4.5 μg of plasmid DNA were used in each well for transfection, including 2 μg of pCTGF-Luc, 0.5 μg of pSV-β-gal, pdn-Tβ-RII at the indicated doses, and the empty vector pcDNA. The amount of pdn-Tβ-RII plus pcDNA was equalized to 2 μg. Cells were treated with or without Cur at 20 μM for 24 h. Luc activity is expressed as relative units after β-galactosidase normalization (n = 6). Values are expressed as means ± SD. *P < 0.05 vs. cells cotransfected with no pdn-Tβ-RII.

![Diagram](http://ajpgi.physiology.org/)

Fig. 5. Interruption of the TGF-β signal transduction pathway by Cur likely occurs at steps downstream of agonists, such as suppressing gene expression of TGF-β receptors. Passaged HSCs were transfected with p3TP-Lux (A and B) or pCTGF-Luc (C and D). Cells in A and C were pretreated with Cur at 20 μM for 24 h before the addition of exogenous active TGF-β1 at the indicated concentrations for an additional 24 h. The total treatment lasted for 48 h. Cells in B and D were simultaneously treated with Cur at 20 μM plus exogenous TGF-β1 at the indicated concentrations for 24 h. Luc activity is expressed as relative units after β-galactosidase normalization (n = 6). *P < 0.05 vs. cells with no treatment (0 μM TGF-β1 + 0 μM Cur). **P < 0.05 vs. cells with Cur at 20 μM without exogenous TGF-β1 (0 μM TGF-β1 + 20 μM Cur).
Curcumin inhibits CTGF expression in HSC

Interruption of the TGF-β signal pathway by curcumin likely occurs at steps downstream from agonists, such as suppressing gene expression of TGF-β receptors. Additional experiments were performed to elucidate the underlying mechanisms of curcumin in the interruption of the TGF-β signaling pathway in activated HSCs. TGF-β signaling is initiated by binding of active TGF-β1 to Tβ-RII, which leads to the phosphorylation and activation of Tβ-RI (5, 35). The latter, in turn, phosphorylates Smad2/3 proteins, which subsequently form a complex with Smad4 and migrate into the nucleus to regulate the expression of target genes, including CTGF (41, 42). We (56) have previously demonstrated that curcumin significantly reduced the gene expression of Tβ-RI and Tβ-RII in passaged HSCs. Curcumin had no apparent impact on the production and activation of latent TGF-β1 (56). We, therefore, hypothesized that interruption of the TGF-β signaling pathway by curcumin likely occurred at steps downstream of agonists, such as suppressing gene expression of TGF-β receptors. If this is true, the addition of exogenous TGF-β1 after pretreatment of cells with curcumin for 24 h is expected to show little impact on activation of TGF-β signaling and stimulation of gene expression of CTGF, because curcumin suppresses gene expression of TGF-β receptors and reduces its bioavailability to the ligand TGF-β1 (56). In great contrast, the simultaneous addition of exogenous TGF-β1 and curcumin is expected to have an apparent effect on activation of TGF-β signaling and stimulation of gene expression of CTGF. To study our hypothesis, HSCs were transfected with the TGF-β-inducible Luc reporter plasmid p3TP-Lux. Cells were then divided into two groups. One group was pretreated with curcumin at 20 μM for 24 h before the addition of exogenous TGF-β1 at various concentrations for an additional 24 h. The other group was simultaneously treated with curcumin at 20 μM plus exogenous TGF-β1 at various concentrations for 24 h. As shown in Fig. 5A, pretreatment of cells with curcumin for 24 h, as expected, interrupted the TGF-β signaling pathway. The subsequent addition of exogenous active TGF-β1 showed no significant role in eliminating the curcumin-inhibitory effect until a very high dose (Fig. 5A). In great contrast, the simultaneous addition of exogenous active TGF-β1 dose dependently abrogated the inhibitory effect of curcumin on TGF-β signaling (Fig. 5B).

To further verify the effect of exogenous TGF-β1 on CTGF gene regulation, HSCs were transfected with the CTGF promoter reporter plasmid pCTGF-Luc. Cells were similarly divided into two groups. As shown in Fig. 5C, pretreatment of cells with curcumin for 24 h, as expected, significantly reduced the promoter activity of CTGF in these cells. The subsequent addition of exogenous TGF-β1 had no significant impact on the inhibitory effect. However, the simultaneous addition of exogenous TGF-β1 with curcumin caused a dose-dependent and significant increase in Luc activity (Fig. 5D), suggesting that exogenous TGF-β1 in this situation stimulated the promoter activity of the CTGF gene. Taken together, our results support the hypothesis that interruption of the TGF-β signaling pathway by curcumin likely occurred at steps downstream of the ligand, such as suppression of gene expression of TGF-β receptors.

Activation of PPAR-γ inhibits gene expression of TGF-β receptors, leading to interruption of TGF-β signaling. We have previously shown that curcumin induces PPAR-γ gene expression and its activity in activated HSCs in vitro. To evaluate the role of PPAR-γ activation in the gene expression of TGF-β receptors, passaged HSCs were treated with the natural PPAR-γ agonist PGJ2 at various concentrations for 24 h. As shown in Fig. 6, A and B, activation of endogenous PPAR-γ by PGJ2 dose dependently suppressed gene expres-

![Image](http://ajpgi.physiology.org/)
sion of Tβ-RI and Tβ-RII at both levels of transcription and translation. Transfection assays of HSCs with the TGF-β receptor promoter reporter plasmids pTβ-RI-Luc or pTβ-RII-Luc confirmed the inhibitory effect of activation of PPAR-γ by PGJ₂ on the gene promoter activity of the two receptors in passaged HSCs (Fig. 6C). The plasmids pTβ-RI-Luc and pTβ-RII-Luc contain, respectively, 965 and 1,670 bp of the 5’-flanking region of the Tβ-RI and Tβ-RII gene promoter subcloned in a Luc reporter plasmid (4). In addition, PGJ₂ caused a dose-dependent reduction in Luc activity in HSCs transfected with p3TP-Lux, suggesting that the activation of PPAR-γ blocked TGF-β signaling in activated HSCs. Taken together, these results indicated that the activation of PPAR-γ inhibited gene expression of TGF-β receptors, leading to interruption of TGF-β signaling in activated HSCs.

*Curcumin significantly increases the level of total cellular GSH.* To further elucidate the underlying mechanisms of curcumin in the inhibition of CTGF gene expression in activated HSCs, we hypothesized that curcumin interrupted TGF-β signaling and inhibited CTGF gene expression by improving the cellular redox and attenuating oxidative stress in activated HSCs. As shown in Fig. 7A, curcumin dose dependently increased the level of total cellular GSH (GSH + GSSG) in passaged HSCs. Inhibition of PPAR-γ activation by its antagonist PD-68235 dramatically reduced the ability of curcumin to increase the level of cellular GSH, suggesting that PPAR-γ activation is a prerequisite for curcumin to increase the level of cellular GSH in activated HSCs. Further analyses indicated that curcumin significantly increased the ratio of GSH to GSSG, a sensitive indicator of oxidant stress, in activated HSCs (Fig. 7B).

*De novo synthesis of GSH plays a pivotal role in interrupting TGF-β signaling and inhibiting CTGF gene expression.* Further experiments were performed to evaluate the role of the increase in the level of GSH in the interruption of TGF-β signaling and inhibition of CTGF gene expression. The level of cellular GSH was altered by the following well-known manipulators of GSH synthesis (2, 50). N-acetylcysteine (NAC) was used to enhance cellular GSH content by supplying cysteine. In contrast, l-buthionine sulfoximine (BSO) was used as a specific inhibitor of glutamate-cysteine ligase (GCL), a critical rate-limiting enzyme in the de novo synthesis of GSH (54). Inhibition of GCL activity by BSO leads to depletion of intracellular GSH (50). As shown in Fig. 8A, NAC, like curcumin, significantly reduced Luc activity in cells transfected with the TGF-β-inducible Luc reporter plasmid p3TP-Lux. Pretreatment of cells with BSO abrogated the effect of curcumin on interrupting TGF-β signaling. These results suggested that simulation of de novo synthesis of GSH played a critical role in the interruption of TGF-β signaling by curcumin. Further experiments demonstrated that NAC, mimicking curcumin, reduced the gene expression of Tβ-RI and Tβ-RII at both levels of transcription and translation in passaged HSCs (Fig. 8, B and C). The inhibitory effect of curcumin on gene expression was eliminated by depletion of cellular GSH in cells treated with BSO (Fig. 8, B and C). BSO itself had little, if any, stimulatory effect on Luc activity and on the expression of genes in passaged HSCs (data not shown). Additional experiments showed that increasing cellular GSH by the GSH precursor NAC significantly reduced, like curcumin, CTGF gene expression at levels of transcription and translation (Fig. 9, A and B). Depletion of cellular GSH by pretreatment of cells with BSO eliminated the inhibitory effect of curcumin on CTGF, suggesting that de novo synthesis of cellular GSH by curcumin might be required for curcumin to inhibit CTGF gene expression in activated HSCs in vitro. Furthermore, the antioxidant NAC, mimicking curcumin, reduced the steady-state level of mRNA and the protein abundance of α(I)-procollagen, a target gene regulated by CTGF, and α-SMA, a marker of activated HSCs. Inhibition of GCL activity by BSO abolished the inhibitory effects of curcumin (Fig. 9). Taken together, these results demonstrated that curcumin significantly increased cellular GSH contents in activated HSCs, which played a critical role in interrupting TGF-β signaling and inhibiting gene expression of CTGF and α(I)-procollagen in activated HSCs.

**DISCUSSION**

During the past decade, advances in the understanding of genes in promoting HSC activation are impressive. However, there have been few breakthroughs in the therapeutic intervention of hepatic fibrogenesis. Therefore, research identifying innocuous antifibrotic agents is of high priority and urgently needed. Most of the evolving antifibrotic therapies are aimed at inhibiting the activation of HSCs, including reducing cell proliferation, inducing apoptosis, and suppressing overproduction of ECM. Our recent studies, including the present report, have addressed this strategy and provided candidates for treatment and prevention of hepatic fibrosis (13, 14, 55, 56). In the
present report, we demonstrated that curcumin dose dependently suppressed the gene expression of CTGF in activated HSCs, which required activation of PPAR-γ. Exogenous CTGF dose dependently abrogated the effect of curcumin on the inhibition of α(I)-collagen gene expression in HSCs. Activation of PPAR-γ by curcumin resulted in the interruption of TGF-β signaling, presumably by suppressing the gene expression of TGF-β receptors, leading to the inhibition of CTGF gene expression. The phytochemical curcumin showed its potent antioxidant property by significantly increasing the level of total GSH and the ratio of GSH to GSSG in activated HSCs. De novo synthesis of cellular GSH was a prerequisite for curcumin to interrupt TGF-β signaling and inhibit gene expression of CTGF and α(I)-collagen in passaged HSCs. Taken together, our results support our initial hypothesis: inhibition of ECM gene expression in activated HSCs by curcumin was mediated by suppressing CTGF gene expression through reducing oxidative stress and interrupting TGF-β signaling.

In the present report, we demonstrated that curcumin at 20 μM significantly inhibited the gene expression of CTGF by ~45–60% and caused a dramatic reduction in the production of α(I)-procollagen by >50% (Figs. 3 and 9). The latter was dose dependently eliminated by exogenous CTGF (Fig. 3), which provides strong support for the conclusion of this report:

Fig. 8. Inhibition of de novo GSH synthesis eliminates the effect of Cur on interrupting TGF-β signaling and suppressing gene expression of TGF-β receptors in activated HSCs. Passaged HSCs were treated for 24 h with N-acetylcysteine (NAC; 5 mM) or Cur (20 μM) or pretreated with the glutamate-cysteine ligase (GCL) inhibitor l-buthionine sulfoximine (BSO; 0.25 mM) for 1 h before the addition of Cur (20 μM). A: Luc assays of HSCs transfected with the TGF-β-inducible reporter plasmid p3TP-Lux. Luc activity is expressed as relative units after β-galactosidase normalization (n = 6). Values are expressed as means ± SD. *P < 0.05 vs. cells with no treatment (Ctr). B: Real-time PCR analyses of the levels of TβRI and TβRII mRNA. mRNA fold changes were calculated by using GAPDH as an invariant control. Values are expressed as means ± SD; n = 3. *P < 0.05 vs. cells with no treatment (Ctr); **P < 0.05 vs. cells treated with Cur. C: Western blot analyses of Tβ-RI and Tβ-RII. β-Actin was used as an internal control for equal loading. Representative images shown are from 3 independent experiments.

Fig. 9. Stimulation of de novo synthesis of GSH by Cur plays a critical role in inhibiting gene expression of CTGF and α(I)-collagen in activated HSCs. Semi-confluent HSCs were treated for 24 h with NAC (5 mM) or Cur (20 μM) or pretreated with the GCL inhibitor BSO (0.25 mM) for 1 h before the addition of Cur (20 μM). Total RNA or whole cell protein extracts were prepared. A: real-time PCR analyses of the mRNA levels of TGF-RI and TGF-RII. mRNA fold changes were calculated by using GAPDH as an invariant control. Values are expressed as means ± SD; n = 3. *P < 0.05 vs. cells with no treatment (Ctr); **P < 0.05 vs. cells treated with Cur. B: Western blot analyses of TGF-RI and TGF-RII. β-Actin was used as an internal control for equal loading. Representative images shown are from 3 independent experiments.
Curcumin suppresses the expression of ECM genes in activated HSCs by inhibiting gene expression of CTGF. These results suggest the possible mechanisms and potential roles of curcumin in the inhibition of the progress of hepatic fibrogenesis in vivo. Overproduction of CTGF in a variety of fibrotic disorders is presumably secondary to the production and activation of TGF-β, a key inducer of ECM synthesis. The promoter of the CTGF gene has a TGF-β response element that regulates its expression (25). We demonstrated that curcumin interrupted TGF-β signaling in activated HSCs in vitro (Fig. 4A), which presumably led to the suppression of CTGF gene expression. This assumption was supported by additional experiments showing that interruption of the TGF-β signaling pathway by forced expression of dominant-negative Tβ-RII resulted in the inhibition of the promoter activity of the CTGF gene (Fig. 4B), mimicking the inhibitory effect of curcumin shown in Fig. 1C.

Curcumin has no apparent inhibitory effect on the production and activation of latent TGF-β in passaged HSCs (56). We, therefore, hypothesized that interruption of TGF-β signaling by curcumin might occur at steps downstream of its agonists, such as receptors, by suppressing gene expression of the receptors and reducing its bioavailability to the ligand TGF-β. Our hypothesis was supported by the observations showing that exogenous TGF-β1 showed differential effects on TGF-β signaling and CTGF gene expression, depending on when cells were exposed to the ligand. The simultaneous addition of TGF-β1 with curcumin dramatically eliminated the inhibitory effects of curcumin on the interruption of TGF-β signaling and inhibition of the promoter activity of the CTGF gene in HSCs (Fig. 5, B and D). In great contrast, the subsequent addition of exogenous TGF-β1 after exposure of cells to curcumin for 24 h could not eliminate the effects of curcumin (Fig. 5, A and C). Treatment of cells with curcumin for 24 h significantly inhibits gene expression of TGF-β receptors in activated HSCs (56). It bears indicating that these results do not exclude possible impacts of curcumin on other downstream steps in the TGF-β signaling pathway.

We (55) have previously shown that curcumin induces the gene expression of PPAR-γ and stimulates its trans-activation activity in activated HSCs in vitro. PPAR-γ agonists are presumed to exist in the media with 10% FBS (29, 36, 56). Activation of PPAR-γ is required for curcumin to inhibit HSC activation, including reduced cell growth and suppressed ECM gene expression (8, 9). In the present report, we observed that blockade of PPAR-γ activation by its antagonist abrogated the inhibitory effect of curcumin on CTGF gene expression (Fig. 1), suggesting that the inhibitory effect might be mediated by the activation of PPAR-γ. The suggestion was supported by additional experiments. As shown in Fig. 2, A and B, activation of endogenous PPAR-γ by PGJ2 dose dependently suppressed gene expression of CTGF at both levels of transcription and translation. In addition, activation of PPAR-γ inhibited gene expression of TGF-β receptors, leading to the interruption of TGF-β signaling in activated HSCs (Fig. 6). The roles of PPAR-γ activation in the suppression of CTGF gene expression and interruption of TGF-β signaling are consistent with another prior report (22). Activation of PPAR-γ inhibits TGF-β-induced CTGF expression by directly interfering with the Smad3 signaling pathway (22).

Curcumin is a potent antioxidant (48), due to both its innate antioxidant chemical structure and its ability to affect enzyme systems involved in the defense against oxidative stress (3, 18). Curcumin inhibits lipid peroxidation (45, 49), nitric oxide synthase activity (9), and production of ROS (31). These unique features might allow curcumin to succeed where other regular antioxidants have failed in the inhibition of hepatic fibrogenesis. In the present report, we demonstrated that curcumin increased the ratio of GSH to GSSG, an indicator of cellular oxidative stress, and the level of total cellular GSH in activated HSCs. The latter required PPAR-γ activation. Depletion of cellular GSH by the GCL inhibitor BSO dramatically eliminated the effects of curcumin on interruption of TGF-β signaling and inhibition of gene expression of CTGF. These results collectively indicated that the level of GSH enhanced by curcumin played a crucial role in inhibiting CTGF gene expression in activated HSC. It remains unknown whether curcumin directly stimulates the activity of preexisting GCL, a rate-limiting enzyme of de novo synthesis of GSH, and/or induces gene expression of GCL in activated HSCs. Other studies (15, 40) have shown that the regulation of many detoxifying enzymes by dietary compounds is mediated by the electrophile response element (EpRE) located in the promoter of the genes. Curcumin induces gene expression of the GCL catalytic subunit in the human branchial epithelial cell line HBE1 by altering EpRE and activator protein-1 binding complexes (18). Our preliminary results suggest that activation of PPAR-γ by curcumin might participate in the induction of gene expression of the GCL catalytic subunit in activated HSCs in vitro (data not shown). Additional experiments are ongoing to elucidate the molecular mechanisms of curcumin in the increase in the level of total cellular GSH in activated HSCs and in the involvement of PPAR-γ activation in the process.

On the basis of our observations, we propose a model to elucidate the underlying mechanisms of curcumin in the inhibition of the production of ECM, including α(I)-collagen, in

![Fig. 10. Schema of the underlying mechanisms of Cur in the reduction of the production of extracellular matrix (ECM), including α(I)-collagen, in activated HSCs in vitro.](http://ajpgi.physiology.org/)
activated HSCs (Fig. 10). The phytochemical curcumin dramatically increases the level of cellular GSH, which requires activation of PPAR-γ. De novo synthesis of GSH by curcumin brings about the interruption of TGF-β signaling by suppressing the gene expression of TGF-β receptors, resulting in inhibition of CTGF gene expression. These effects ultimately lead to a reduction in the production of ECM, including α(I)-collagen, in activated HSCs in vitro. It bears emphasis that our results and this model do not exclude any other possible mechanisms of curcumin in the suppression of CTGF gene expression and reduction of ECM production in activated HSCs. Results generated from our studies provide novel insights into the roles and mechanisms of curcumin in the inhibition of HSC activation. The unique characteristics of curcumin, including the potent capability to attenuate oxidative stress, inducing gene expression of endogenous PPAR-γ, inhibiting HSC activation, and no adverse health effects, might make it a good antioxidant candidate for the treatment and prevention of hepatic fibrosis.

GRANTS

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