

# Curcumin attenuates the effects of insulin on stimulating hepatic stellate cell activation by interrupting insulin signaling and attenuating oxidative stress

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Hyperinsulinemia associated with type II diabetes mellitus (T2DM) is a risk factor for non-alcoholic steatohepatitis (NASH) and hepatic fibrosis. Hepatic stellate cells (HSCs) are the major effectors in collagen production during hepatic fibrogenesis. Elevated levels of insulin stimulate HSC activation. In addition to its anti-diabetic effects, the antioxidant curcumin, the yellow pigment in curry from turmeric, suppresses HSC activation and protects the liver from fibrogenesis *in vitro* and *in vivo*. This study aims at evaluating the effect of curcumin on insulin-induced HSC activation and further elucidating the underlying mechanisms. We report that curcumin dose-dependently eliminates insulin-induced HSC activation by suppressing expression of type I collagen gene and other key genes relevant to HSC activation. Additional experiments indicate that curcumin interrupts insulin signaling in HSCs by reducing the phosphorylation level of insulin receptor (InsR) and suppressing gene expression of InsR. Furthermore, curcumin attenuates insulin-induced oxidative stress in HSCs by inducing gene expression of glutamate-cysteine ligase (GCL), leading to *de novo* synthesis of glutathione and the suppression of gene expression of InsR. These results support our initial hypothesis that curcumin inhibits the effects of insulin on stimulating HSC activation by interrupting insulin signaling and attenuating oxidative stress. Our results provide novel insights into the mechanisms by which curcumin inhibits the insulin-induced HSC activation.

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Hyperinsulinemia, that is, abnormally elevated levels of plasma insulin, is a feature of type II diabetes mellitus (T2DM). T2DM is recognized as the etiology of over 80% of all diabetes and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index.<sup>1</sup> Hyperinsulinemia is a condition in which normal amounts of insulin could not produce a normal insulin response from fat, muscle and liver cells.<sup>1,2</sup> As a compensatory mechanism, pancreatic beta-cells excessively secrete insulin to maintain euglycemia, resulting in abnormally elevated levels of blood insulin.<sup>3</sup> Insulin in normal state carries out its functions through binding to insulin receptor (InsR), leading to the auto-phosphorylation of InsR and the activation of downstream signaling cascades, including the

mitogen-activated protein kinase (MAPK) pathway and the PI3K–AKT pathway.<sup>4</sup>

The liver is an insulin-sensitive organ that has a critical role in regulating the whole-body energy homeostasis.<sup>2</sup> It has been noted that T2DM is often associated with non-alcoholic fatty liver diseases (NAFLDs) and is a risk factor for non-alcoholic steatohepatitis (NASH), an advanced form of NAFLD.<sup>5</sup> NAFLDs are found in a quarter of the general population in the United States, and approximately 15–40% of NASH patients develop hepatic fibrosis.<sup>6</sup> Hepatic stellate cells (HSCs) are the key players in the development of hepatic fibrosis, regardless of etiology.<sup>7,8</sup> HSCs normally lie in the space of Disse in a quiescent, non-proliferative state. They are characterized by abundant lipid droplets, composed of retinyl

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esters, triglycerides, cholesteryl esters, cholesterol, phospholipids and free fatty acids.<sup>9,10</sup> During hepatic injury, quiescent HSCs undergo profound phenotypic changes, including enhanced cell proliferation, loss of lipid droplets, *de novo* expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and excessive production of ECM. This process is called HSC activation. Freshly isolated HSCs in culture gradually and spontaneously become fully activated in 7 days,<sup>11</sup> mimicking the process observed *in vivo*, thus providing a good model for elucidating the underlying mechanisms of HSC activation and studying potential therapeutic intervention of the process.<sup>7,8</sup> Studies have shown that insulin stimulates HSC activation *in vitro* by inducing mitogenesis and collagen synthesis.<sup>12</sup>

Despite considerable accomplishments in research on NASH-associated hepatic fibrogenesis, the underlying mechanisms remain largely undefined. It is widely accepted that oxidative stress has critical roles in hepatic fibrosis, regardless of etiology.<sup>13</sup> For instance, during the pathogenesis of NASH, fat accumulation in the liver is considered as 'the first hit',<sup>1</sup> which makes the liver vulnerable to endotoxins and impairs liver regeneration. Oxidative stress is recognized as 'the second hit',<sup>1</sup> which causes peroxidation of lipids in cell membranes, pro-inflammatory cytokine induction and the activation of HSCs. NASH patients have increased levels of oxidative stress and lipid peroxidation products,<sup>1,2</sup> which, in turn, promotes the development of hepatic fibrogenesis.<sup>1,2</sup> The activities of antioxidant enzymes in NASH patients are dramatically reduced.<sup>14</sup> Oxidative stress stimulates collagen production in HSCs and hepatic fibrogenesis.<sup>14</sup> Previous reports have shown protective effects of antioxidants, including vitamin E, in the suppression of HSC activation<sup>13</sup> and the inhibition of hepatic fibrogenesis.<sup>13</sup> However, the efficiency of currently well-known antioxidants in protecting the liver from fibrogenesis is still not very impressive.<sup>13,15</sup>

Few effective therapies are currently available for treatment of hepatic fibrosis.<sup>16</sup> Research identifying anti-fibrotic agents that are innocuous is, therefore, of high priority and urgently needed. Curcumin, the yellow pigment in curry from turmeric, is a potent antioxidant, whose antioxidant capacity is 100-fold stronger than vitamin E/C.<sup>17</sup> Curcumin has received attention as a promising dietary component for the protection against fibrogenic insults.<sup>18</sup> We recently showed that curcumin inhibited HSC activation, including inducing gene expression of endogenous peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and suppressing gene expression of  $\alpha$ 1(I) collagen,  $\alpha$ -SMA, PDGF- $\beta$  receptor (PDGF- $\beta$ R), EGF receptor (EGFR), type I and II transforming growth factor- $\beta$  receptors (T $\beta$ -RI and T $\beta$ -RII) and connective tissue growth factor (CTGF), and protected the liver from CCl<sub>4</sub>-caused fibrogenesis *in vitro* and *in vivo*.<sup>18–22</sup> The current study aims at evaluating the effect of curcumin on eliminating the role of insulin in the stimulation of HSC activation and further elucidating the underlying mechanisms. Results in this report provide evidence to support our initial hypothesis that curcumin inhibits the effects of insulin on stimulating HSC

activation by interrupting insulin signaling and attenuating oxidative stress.

## MATERIALS AND METHODS

### Materials

Insulin and curcumin (purity >94%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Insulin was dissolved in 0.01 N HCl and a stocking concentration at 1 mM was prepared. Curcumin was dissolved in 100% ethanol and a stocking concentration at 100 mM was made.

### HSC Isolation and Culture

Hepatic stellate cells were isolated from the liver of normal male Sprague-Dawley rats (200–250 g) through portal vein perfusion with collagenase-pronase and subsequent density gradient centrifugation as previously described.<sup>11,20</sup> After isolation, HSCs were identified by their typical morphology, the vitamin A-droplet-dependent auto-fluorescence and their inability to phagocytose latex beads. Freshly isolated HSCs were plated on plastic plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) at 37 °C in a 5% CO<sub>2</sub> atmosphere for the first 48 h. The purity of HSCs was greater than 95%, as assessed at 48 h after seeding. HSCs were subsequently cultured in regular DMEM with FBS (10%). Semi-confluent HSCs with four–eight passages were used in experiments. In some experiments, cells were cultured in serum-depleted media for 24 h before treatment, which rendered HSCs more sensitive to stimuli, including exogenous insulin. Cells were subsequently treated and cultured in serum-depleted media for additional 24 h, which excluded the interference from other factors in FBS.

### Oil Red O Staining

After fixation with 4% paraformaldehyde for 15 min, HSCs were stained with Oil Red O in isopropanol (60%) for 10 min, followed by differentiation with isopropanol (60%) for 2 min. The cells were counterstained with hematoxylin for 15 s and washed with distilled water thoroughly. Lipid droplets in HSCs were evaluated using a light microscope with  $\times 40$  amplification. Lipid droplets in quiescent HSCs were colored dark red by Oil Red O.

### mRNA Extraction and Real Time PCR

Total RNA was extracted using TRI reagent according to the manufacturer's instruction (Sigma). Total RNA was treated with DNase I before the synthesis of the first strand of cDNA. Real-time PCR were performed as we previously described using SYBR Green Supermix.<sup>23</sup> mRNA levels were expressed as fold changes after normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described by Schmittgen *et al.*<sup>24</sup> The following primers were used for real-time PCR. *InsR*: (F) 5'-GCCTGGGCAACTGTTTCAGA-3', (R) 5'-GTTTCGACAGGCCACACACTT-3'. *Gcl*: (F) 5'-TGTGTGATGAGCCCAAGGAC-3'; (R) 5'-AGTTGGCTCGCATCATAGTTG-3'; *Gclm*: (F) 5'-CTGCTAAACTGTTTCAT

TGTAGG-3'; (R) 5'-CTATGGGTTTACCTGTG-3'. Other primers were recently described.<sup>25</sup>

### Western Blotting Analyses

Whole cell extracts were prepared as we previously described.<sup>20</sup> Protein concentrations were determined using the BCA Protein Assay Kit according to the manufacturer's instruction (Pierce, Rockford, IL, USA). SDS-PAGE, transblotting and subsequent immunoreactions were conducted as we previously described.<sup>20</sup> The antibodies used in this study are described in Table 1.

### Determination of the Level of Intracellular Reactive Oxygen Species (ROS)

The level of intracellular ROS in HSCs was determined by analyzing dichlorofluorescein (DCF) fluorescence, as described previously.<sup>26</sup>

**Table 1 Antibodies used for Western blotting analyses**

Company	Description	Catalog number
Santa Cruz	Rabbit $\alpha$ -PDGF- $\beta$ R antibody	sc-432
Biotechnology, Inc.	Rabbit $\alpha$ -EGFR antibody	Sc-03
	Rabbit $\alpha$ -T- $\beta$ -RII antibody	Sc-400
	Rabbit $\alpha$ -T- $\beta$ -RI antibody	Sc-399
	Rabbit $\alpha$ -p27 antibody	Sc-528
	Rabbit $\alpha$ -p21 antibody	Sc-397
	Rabbit $\alpha$ -Bax antibody	Sc-493
	Rabbit $\alpha$ -Bcl-2 antibody	Sc-492
	Rabbit $\alpha$ -InsR antibody	Sc-20739
	Rabbit $\alpha$ -p-InsR antibody	Sc-25103-R
	Rabbit $\alpha$ -p-ERK1/2 antibody	Sc-16982-R
	Rabbit $\alpha$ -total ERK1 antibody	Sc-94
	Rabbit $\alpha$ -total ERK2 antibody	Sc-153
	Rabbit $\alpha$ -p-AKT <sup>ser 473</sup> antibody	Sc-7985-R
	Rabbit $\alpha$ -total AKT antibody	Sc-8312
	Rabbit $\alpha$ -p-JNK antibody	Sc-12882-R
	Rabbit $\alpha$ -total JNK antibody	Sc-474
	Rabbit $\alpha$ -p-PI3K antibody	Sc-12929-R
	Rabbit $\alpha$ -total PI3K antibody	Sc-423
	Goat $\alpha$ -CTGF antibody	Sc-14939
	Goat $\alpha$ -pro- $\alpha$ (I)col antibody	Sc-25974
Sigma	Bovine $\alpha$ -goat-IgG-HRP	Sc-2350
	Goat $\alpha$ -mouse-IgG-HRP	Sc-2005
	Goat $\alpha$ -rabbit-IgG-HRP	Sc-2004
	Mouse $\alpha$ -alpha-SMA monoclonal antibody	A2547
	Rabbit $\alpha$ - $\beta$ -actin antibody:	A2066

### Lipid Peroxide (LPO) Assays

LPO assays were performed using the lipid hydroperoxide assay kit purchased from Cayman Chemical (Ann Arbor, MI, USA).

### Glutathione (GSH) Assays

The levels of GSH and GSSG were determined by the glutathione assay kit from Cayman Chemical, following the protocol provided by the manufacturer. The concentration of total GSH was calculated according to the equation in the protocol.

### Analyses of Glutamate-Cysteine Ligase (GCL) Activities

Glutamate-cysteine ligase activities were spectrophotometrically determined as previously described with slight modifications.<sup>27</sup> In brief, a sample of cell extracts (20  $\mu$ l) was mixed with the reaction solution (0.21 ml) containing Tris-HCl (100 mM), pH 8.0, KCl (150 mM), MgCl<sub>2</sub> (20 mM), Na<sub>2</sub>EDTA (2 mM), Na<sub>2</sub>ATP (5 mM), phosphoenolpyruvate (2 mM), L-glutamate (10 mM), L- $\alpha$ -aminobutyrate (10 mM), NADH (0.27 mM), type II rabbit muscle pyruvate (2  $\mu$ g) and lactate dehydrogenase (2  $\mu$ g). The reaction was initiated by the addition of ATP to a final concentration at 5 mM. The decrease in the absorbance of NADH at 340 nm was monitored for 30 min with an interval of 2 min using a SpectraMax 190 plate reader (Molecular Device, Sunnyvale, CA, USA) and expressed as  $\mu$ mol of NADH oxidized/min. Protein concentration was quantitated using BCA assay (Pierce). The final GCL activity was calculated and expressed as  $\mu$ mol of NADH oxidized/min per mg protein.

### Statistical Analyses

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

## RESULTS

### Curcumin Eliminates the Effects of Insulin on Regulating Expression of Genes Closely Relevant to HSC Activation in Culture-Activated HSCs

Previous studies have shown that insulin stimulates HSC activation *in vitro* by inducing mitogenesis and collagen synthesis.<sup>12</sup> To evaluate the effect of curcumin on insulin-induced HSC activation, semi-confluent HSCs, after being cultured in serum-depleted media for 24 h, were stimulated with insulin (100 nM) in the presence of curcumin at 0–30  $\mu$ M in serum-depleted DMEM for additional 24 h. Results from our pilot experiments indicated that compared with serum-starved HSCs, HSCs cultured in regular DMEM with FBS (10%) required higher concentrations of insulin to achieve the same level of changes in regulating expression of genes, including  $\alpha$ I(I) collagen and  $\alpha$ -SMA, the two established markers for activated HSCs (data not shown). These observations suggested that serum starvation rendered HSCs

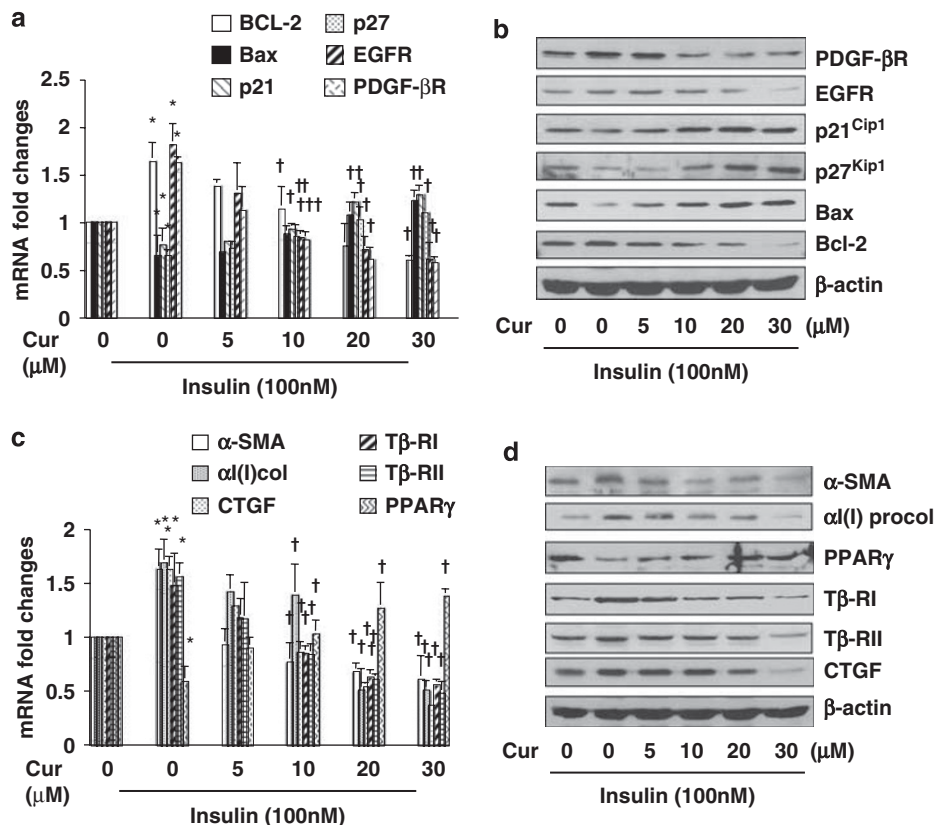
*in vitro* more sensitive to exogenous stimuli. The subsequent culture in serum-depleted media excluded the interference from other factors in FBS.<sup>21,28</sup> Total RNA and whole cell extracts were prepared from the cells. To evaluate the effects of curcumin on insulin-induced cell growth, genes relevant to cell proliferation and to apoptosis were selectively studied. As shown by real-time PCR assays (Figure 1a), compared with the untreated control (the corresponding first columns), insulin significantly increased, as expected, the mRNA levels of pro-mitogenic PDGF- $\beta$ R and EGFR (the corresponding second columns), and reduced the mRNA levels of the potent cyclin-dependent kinase inhibitors p21<sup>Cip1</sup> and p27<sup>Kip1</sup> (the corresponding second columns). In addition, insulin increased the mRNA level of anti-apoptotic protein Bcl-2 and reduced the mRNA level of pro-apoptotic protein Bax in the cells (the corresponding second columns). Further experiments indicated that curcumin dose-dependently eliminated the insulin effects (the corresponding 3–6 columns). These observations were verified using Western blotting analyses (Figure 1b).

To assess the effect of curcumin on insulin-induced fibrogenesis, genes relevant to fibrogenesis were selectively

studied. Real-time PCR (Figure 1c) and Western blotting analyses (Figure 1d) showed that compared with the untreated control (the corresponding first columns or wells), insulin stimulated, as expected, gene expression of  $\alpha$ -SMA and  $\alpha$ I(I) collagen, the markers of activated HSCs, and pro-fibrogenic T $\beta$ -RI and T $\beta$ -RII and CTGF (the corresponding second columns or well). The stimulatory effects of insulin were dose-dependently attenuated by curcumin (the corresponding 3–6 columns). It is noteworthy that insulin suppressed gene expression of PPAR $\gamma$  in cultured HSCs (Figures 1c and d). Curcumin dose-dependently abrogated the inhibitory role of insulin and induced gene expression of endogenous PPAR $\gamma$ , which had been shown to have a critical role in the curcumin-caused inhibition of HSC activation.<sup>20,21</sup> Taken together, our results suggested that curcumin eliminated the effects of insulin on stimulating HSC activation *in vitro*.

### Insulin Stimulates the Activation of Quiescent HSCs *In Vitro*, Which is Attenuated by Curcumin

To evaluate the effect of insulin on the activation of quiescent HSCs and the potential inhibitory role of curcumin in the



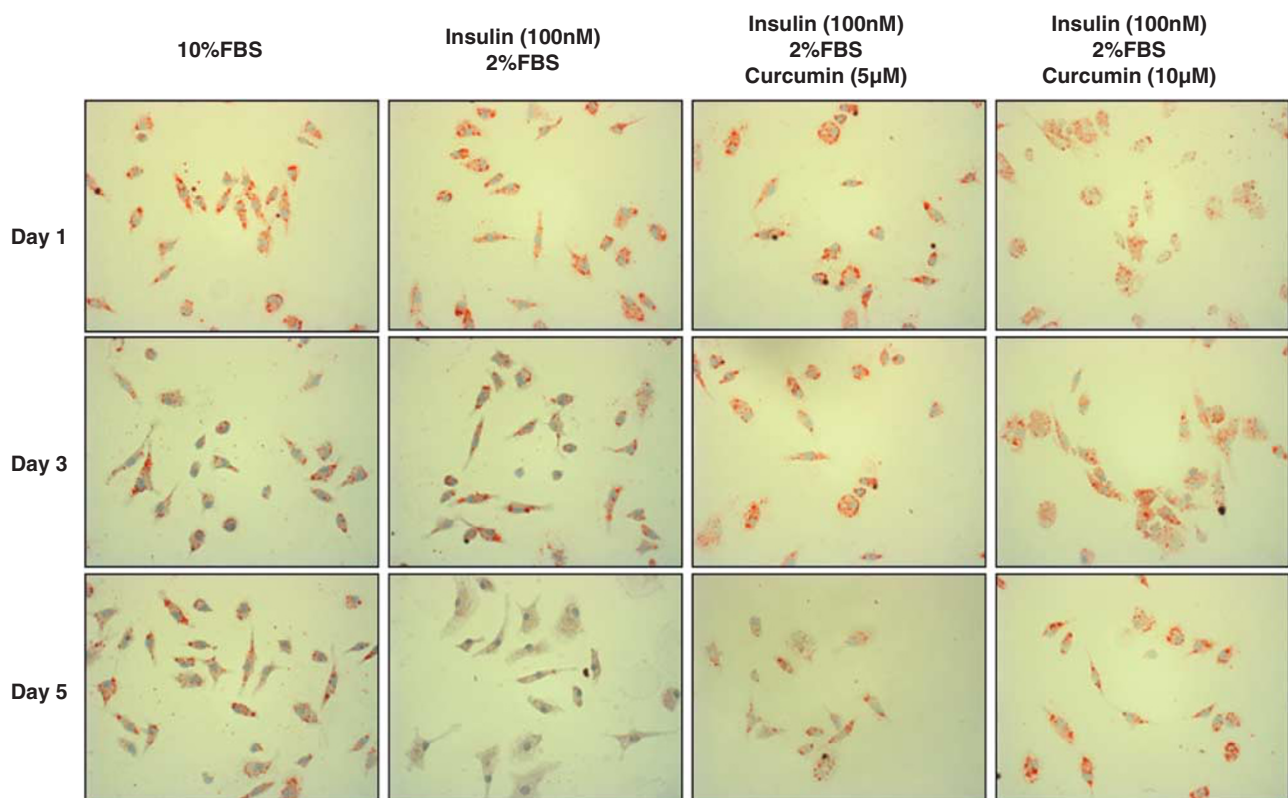
**Figure 1** Curcumin attenuates the stimulatory effects of insulin on the activation of HSCs. Serum-starved HSCs were stimulated with or without insulin (100 nM) and curcumin at various concentrations in serum-depleted DMEM for 24 h. Total RNA or whole cell extracts were prepared for real-time PCR assays (**a**, **c**), or for Western blotting analyses (**b**, **d**). Values in **a** and **c** were presented as mRNA fold changes (mean  $\pm$  s.d.,  $n = 3$ ), \* $P < 0.05$ , versus the untreated control (the corresponding first columns);  $^{\dagger}P < 0.05$ , versus cells treated with insulin only (the corresponding second columns). Blots were representatives from three independent experiments.  $\beta$ -actin was used as an internal control for equal loading for Western blotting analyses. (**a**, **b**) Analyses of expression of genes relevant to cell proliferation or apoptosis and (**c**, **d**) analyses of expression of genes relevant to fibrogenesis.

process, additional experiments were conducted. After culturing for 24 h in serum-rich media (20%FBS) for cell attachment, freshly isolated quiescent HSCs were cultured in DMEM with 10%FBS, or 2%FBS and insulin (100 nM) with or without curcumin at 5 or 10  $\mu$ M for additional 1, 3 or 5 days. These HSCs were stained with Oil Red O for detecting intracellular lipid droplets. As shown in Figure 2, after 1-day culture, regardless of the supplements in the media with or without curcumin, HSCs showed high levels of intracellular lipid droplets, which were stained dark red by Oil Red O, demonstrating that these HSCs were still in quiescent state. Insulin (100 nM) and 2% FBS, similar to FBS (10%), day-dependently and apparently reduced the contents of lipid droplets in HSCs, suggesting that insulin, similar to FBS, stimulated the activation of HSCs in culture. Most of freshly isolated quiescent HSCs were detached within 3 days in the media with only 2% FBS (data not shown). In great contrast, the presence of curcumin in the media with insulin and 2% FBS dose-dependently maintained higher levels of intracellular lipid droplets after 5 days of culture, suggesting that curcumin attenuated the stimulatory role of insulin, which might delay the process of HSC activation in culture. Lower concentrations of curcumin at 5 and 10  $\mu$ M were used

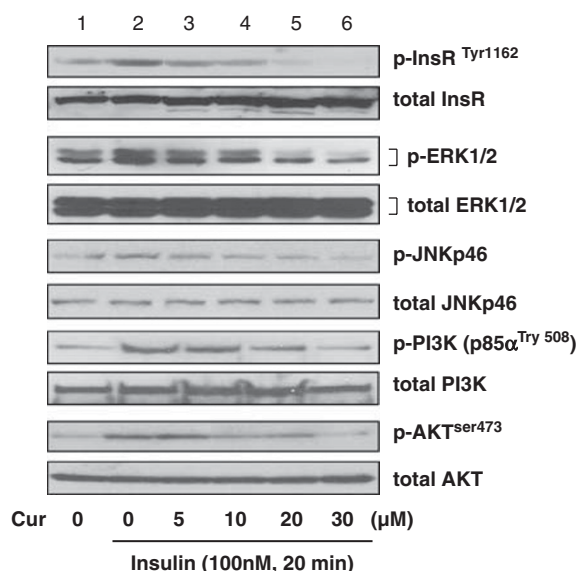
because of the higher sensitivity of freshly isolated HSCs to the phytochemical. Most of the cultured cells were detached within 7 days in the media with insulin (100 nM) and 2% FBS in the presence of curcumin (data not shown). Quiescent HSCs were reported to become fully activated after 7 days of culture in DMEM with 10% FBS.<sup>11</sup> However, the technical barrier prevented us from conducting the experiments for more than 5 days. Taken together, our results showed that insulin stimulated the activation of quiescent HSCs *in vitro*, which was attenuated by curcumin.

### Curcumin Reduces the Phosphorylation of InsR and Its Downstream Inter-Mediators in HSCs

To explore the underlying mechanisms by which curcumin eliminated the insulin-induced HSC activation, we hypothesized that curcumin might interrupt insulin signaling pathways, which was initiated by binding of insulin to its receptor InsR, leading to the auto-phosphorylation of the receptor and the subsequent activation of downstream signaling inter-mediators, including ERK, JNK, PI3K and AKT.<sup>4</sup> Pilot experiments indicated that insulin rapidly initiated its signaling by stimulating the phosphorylation of InsR in HSCs, which reached its peak within 20–30 min and



**Figure 2** Insulin stimulates the activation of quiescent HSCs *in vitro*, which is attenuated by curcumin. After culturing for 24 h in serum-rich media (20%FBS) for cell attachment, freshly isolated HSCs were cultured in DMEM with 10% FBS, or 2% FBS and insulin (100 nM) with or without curcumin at indicated concentrations for additional 1, 3 or 5 days. Each treatment had triplicates. After fixation, the cells were stained with Oil Red O for detecting intracellular lipid droplets, which were colored dark red by Oil Red O. Representative views were presented.

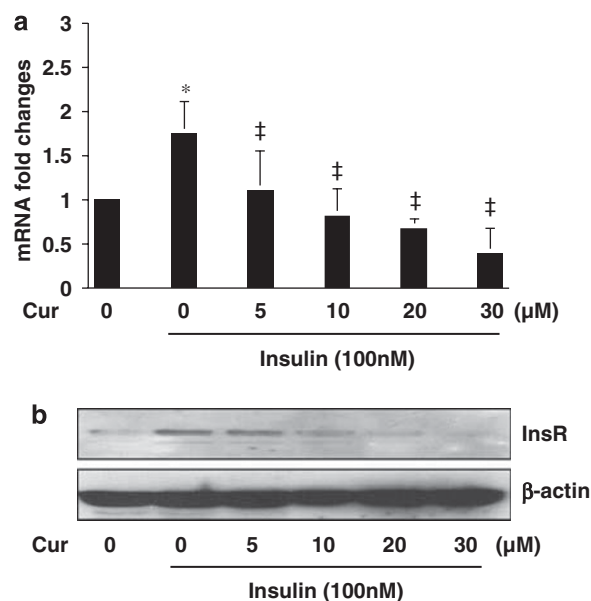


**Figure 3** Curcumin reduces phosphorylation levels of InsR and its downstream signaling inter-mediators in HSCs. Serum-starved HSCs were pretreated with curcumin for 1 h at various concentrations before stimulation with insulin (100 nM) for additional 20 min. Whole cell extracts were prepared for Western blotting analyses of phosphorylated proteins and their corresponding total proteins. The total protein for each corresponding phospho-protein was used as the internal control for equal loading. Representatives were shown from three independent experiments.

gradually faded thereafter (data not shown here). To evaluate the effect of curcumin on insulin signaling pathways, serum-starved HSCs were pretreated with curcumin at various concentrations for 1 h before stimulation with insulin (100 nM) in serum-depleted DMEM for additional 20 min. The pretreatment with curcumin inhibited activating signal pathways and excluded their interference. Whole cell extracts were prepared. As shown in Figure 3, using Western blotting analyses insulin significantly increased the abundance of phosphorylated InsR<sup>Tyr 1162</sup>, ERK1/2, JNKp46, PI3K (p85α<sup>Tyr 508</sup>) and AKT<sup>ser 473</sup> (the corresponding second wells) in cultured HSCs when compared with the untreated control (the corresponding first wells). The insulin stimulatory effects were dose-dependently attenuated by curcumin (the corresponding 2–6 wells), indicating that curcumin interrupted the insulin signaling pathways. It is noteworthy that the phosphorylation of InsR and its downstream inter-mediators was an instant and short-term action after insulin stimulation. The effect of curcumin on it was a short-term event, which lasted only for a few hours.

### Curcumin Dose-Dependently Suppresses Gene Expression of InsR in Cultured HSCs

To further explore the mechanisms by which curcumin eliminated the stimulatory effects of insulin on the activation of HSCs, we assumed that in addition to the instant and short-term action, curcumin should exert a slow but long-lasting action. We postulated that curcumin suppressed gene



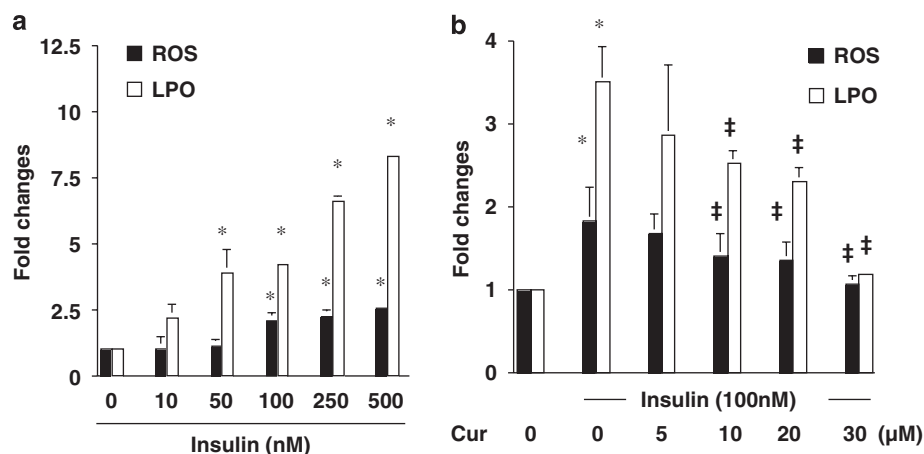
**Figure 4** Curcumin dose-dependently suppresses gene expression of InsR in cultured HSCs. Serum-starved HSCs were stimulated with or without insulin (100 nM) in the presence or absence of curcumin at indicated concentrations in serum-free media for 24 h. Total RNA or whole cell extracts were prepared. (a) Real-time PCR assays of the steady-state level of InsR mRNA.  $\beta$ -actin was used as an invariant internal control for calculating mRNA fold changes ( $n = 3$ ). \* $P < 0.05$  versus cells with no treatment (the first column on the left); ‡ $P < 0.05$  versus cells treated with insulin only (the second column on the left). (b) Western blotting analyses of the abundance of InsR.  $\beta$ -actin was used as an internal control for equal loading. A representative was shown from three independent experiments.

expression of InsR, leading to a reduction in the bioavailability of InsR to insulin. To test this postulation, serum-starved HSCs were stimulated with insulin at 100 nM in the presence of curcumin at 0–30  $\mu$ M in serum-depleted media for 24 h. Total RNA and whole cell extracts were prepared for analyses of gene expression of InsR. As shown in Figure 4, using real-time PCR and Western blotting analyses insulin significantly increased the steady-state level of transcript and the abundance of protein of InsR (the second column or well) when compared with the untreated control (the first column or well). The stimulatory effects were dose-dependently abolished by curcumin (the 3–6 columns or wells). Taken together, these results supported our postulation and showed that curcumin suppressed gene expression of InsR in activated HSCs *in vitro*.

### Insulin Increases the Levels of Intracellular ROS and LPO in HSCs, Which is Dose-Dependently Attenuated by Curcumin

Oxidative stress has been known to have a critical role in HSC activation and in hepatic fibrogenesis, regardless of etiology.<sup>13,29</sup> We have previously reported the role of curcumin alone in attenuating oxidative stress in cultured HSCs.<sup>23</sup> To explore the mechanisms by which insulin stimulated the





**Figure 5** Insulin stimulates oxidative stress in cultured HSCs by increasing the levels of ROS and LPO, which is attenuated by curcumin. Serum-starved HSCs were stimulated with insulin at indicated concentrations in the absence (a) or presence (b) of curcumin at 0–30  $\mu$ M in serum-free media for 24 h. The levels of intracellular ROS and LPO were assessed and expressed as fold changes compared with cells with no treatment (the first column on the left). Values were presented as means  $\pm$  s.d. ( $n \geq 3$ ). \* $P < 0.05$ , versus cells with no treatment; † $P < 0.05$ , versus cells treated with insulin only (the second column on the left in b).

activation of HSCs, experiments were conducted to evaluate the effect of insulin on oxidative stress. Serum-starved HSCs were stimulated with different concentrations of insulin in serum-depleted media for 24 h. The levels of LPO and ROS in the cells were determined. As shown in Figure 5a, insulin elevated the levels of cellular ROS and LPO in a dose-dependent manner. To assess the role of curcumin in attenuating insulin-induced oxidative stress, serum-starved HSCs were stimulated with insulin (100 nM) in the presence or absence of curcumin at indicated concentrations in serum-depleted media for 24 h. As shown in Figure 5b, ROS and LPO levels elevated by insulin in the cells were dose-dependently reduced by curcumin. These results collectively indicated that insulin induced oxidative stress in cultured HSCs, which was attenuated by curcumin.

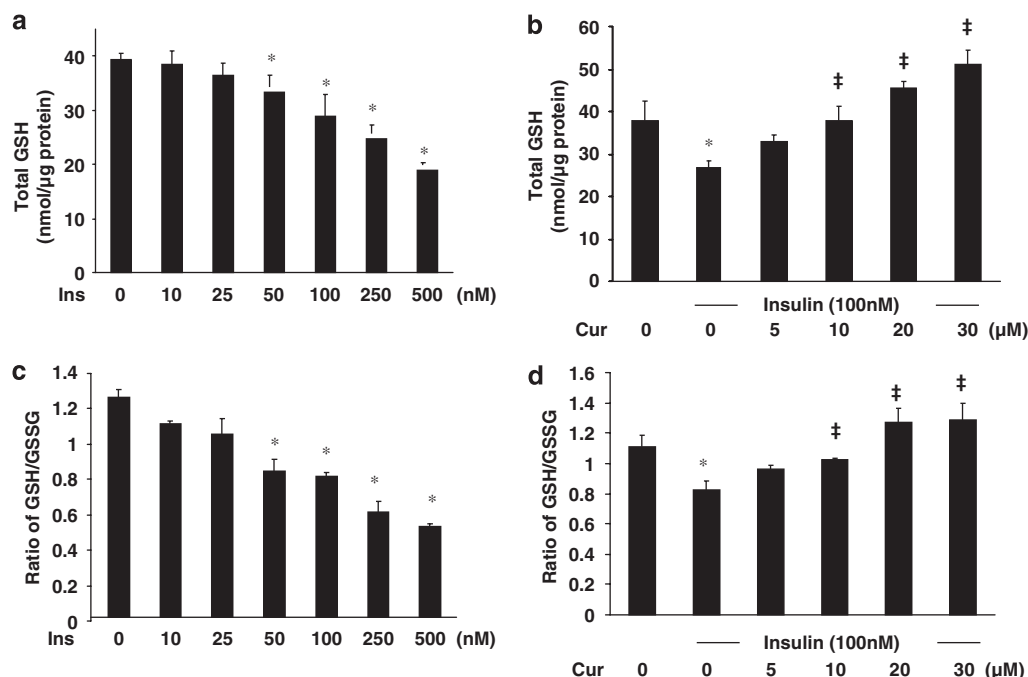
#### Curcumin Attenuates Insulin-Induced Oxidative Stress in Activated HSCs, at Least Partially, by Increasing the Level of Intracellular GSH and Improving the Ratio of GSH/GSSG

Glutathione is the most abundant and effective thiol antioxidant in eukaryotic cells.<sup>30</sup> GSH is converted to its oxidized form (GSSG), when attenuating oxidative stress.<sup>30</sup> To elucidate the mechanisms by which insulin induced oxidative stress and curcumin attenuated the insulin-induced oxidative stress, serum-starved HSCs were stimulated with insulin at indicated concentrations in the presence or absence of curcumin at various concentrations in serum-depleted media for 24 h. As shown in Figures 6a and c, insulin reduced, in a dose-dependent manner, the levels of intracellular GSH and the ratio of GSH/GSSG, a sensitive indicator of oxidant stress.<sup>31,32</sup> On the other hand, curcumin dose-dependently

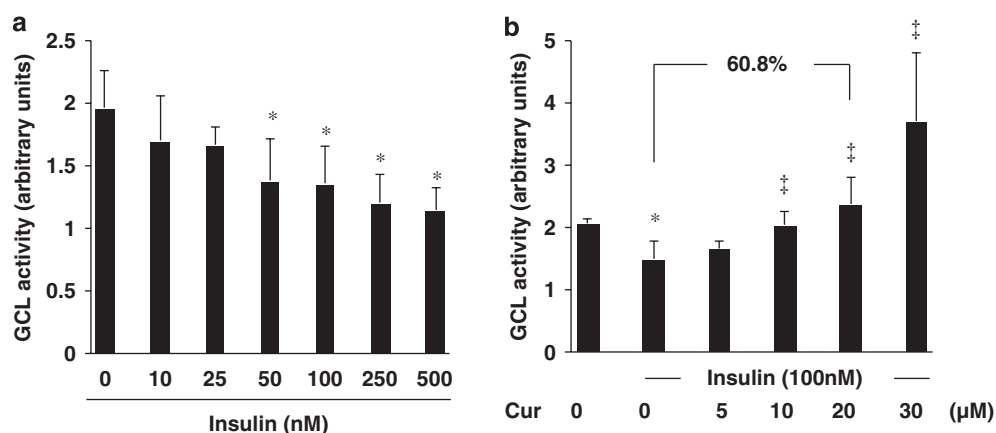
eliminated the inhibitory effects of insulin on the level of cellular GSH (Figure 6b) and improved the ratio of GSH/GSSG (Figure 6d). These results indicated that curcumin attenuated insulin-induced oxidative stress in activated HSCs, at least partially, by increasing the level of intracellular GSH and improving the ratio of GSH/GSSG.

#### Curcumin Increases the Level of Cellular GSH and Attenuates Insulin-Induced Oxidative Stress in Activated HSCs *In Vitro* by Stimulating the Activity of GCL

The level of cellular GSH is mainly determined by GSH synthesis (GSH supply) and GSH-consuming (GSH demand). GCL is the key rate-limiting enzyme in *de novo* synthesis of GSH.<sup>27</sup> To understand the mechanisms by which insulin reduced the levels of cellular GSH and curcumin eliminated the inhibitory effects, we assumed that insulin might reduce the GCL activity in HSCs, which was eliminated by curcumin. To test the assumption, serum-starved HSCs were stimulated with insulin at indicated concentrations in the presence or absence of curcumin at various doses in serum-free media for 24 h. Whole cell extracts were prepared for analyzing the GCL activity. The results in Figure 7a showed that insulin dose-dependently reduced the activity of GCL in these cells. The inhibitory effect of insulin was eliminated by curcumin (Figure 7b). For example, compared with the control cells treated with insulin only (the second column in Figure 7b), curcumin at 20  $\mu$ M abrogated the inhibitory effect of insulin and caused a significant increase in the activity of GCL by 60.8%. Taken together, these results supported our assumption and showed that curcumin increased the level of cellular GSH and attenuated insulin-induced oxidative stress in activated HSCs *in vitro* by stimulating the activity of GCL.



**Figure 6** Insulin reduces the level of intracellular GSH and the ratio of GSH/GSSG in cultured HSCs, which are dose-dependently eliminated by curcumin. Serum-starved HSCs were stimulated with insulin at indicated concentrations in the absence (a, c) or presence (b, d) of curcumin at 0–30 μM in serum-free media for 24 h. The levels of total cellular GSH (a, b) and the ratio of GSH/GSSG (c, d) were determined. The levels of total GSH were expressed as nmol/μg protein. Values were presented as means ± s.d. ( $n \geq 3$ ). \* $P < 0.05$ , versus cells with no treatment (the first column on the left). ‡ $P < 0.05$ , versus cells treated with insulin only (the second column on the left in b, d).



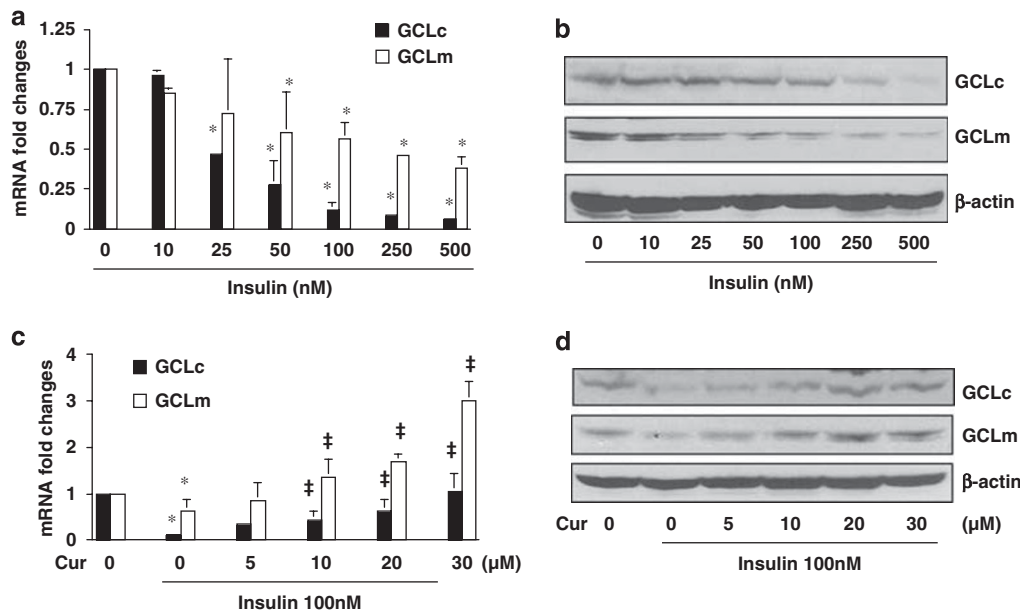
**Figure 7** Curcumin eliminates the role of insulin in the reduction of the activity of GCL in activated HSCs *in vitro*. Serum-starved HSCs were stimulated with insulin at indicated concentrations in the absence (a) or presence (b) of curcumin at 0–30 μM in serum-free media for 24 h. The activities of GCL in the cells were determined. Values were expressed as means ± s.d. ( $n \geq 6$ ). The number of percentage indicates the increase in the GCL activity in cells treated with insulin (100 nM) and curcumin (20 μM) compared with cells treated with insulin only. \* $P < 0.05$ , versus cells with no treatment (the first column on the left). ‡ $P < 0.05$ , versus cells treated with insulin only (the second column on the left in b).

### Curcumin Increases the Activity of GCL and the Level of Cellular GSH in Insulin-Activated HSCs by Inducing Gene Expression of GCLc and GCLm

The enzyme of GCL is composed of a large catalytic subunit (GCLc, ~73 kDa) and a small modulatory subunit (GCLm, ~30 kDa). To answer the question of how curcumin could

increase the activity of GCL and elevate the level of cellular GSH in insulin-activated HSCs, we postulated that curcumin might induce gene expression of the GCL subunits GCLc and GCLm, leading to the increase in the GCL activity and the elevation of the level of cellular GSH. To test the postulation, serum-starved HSCs were stimulated with insulin at various





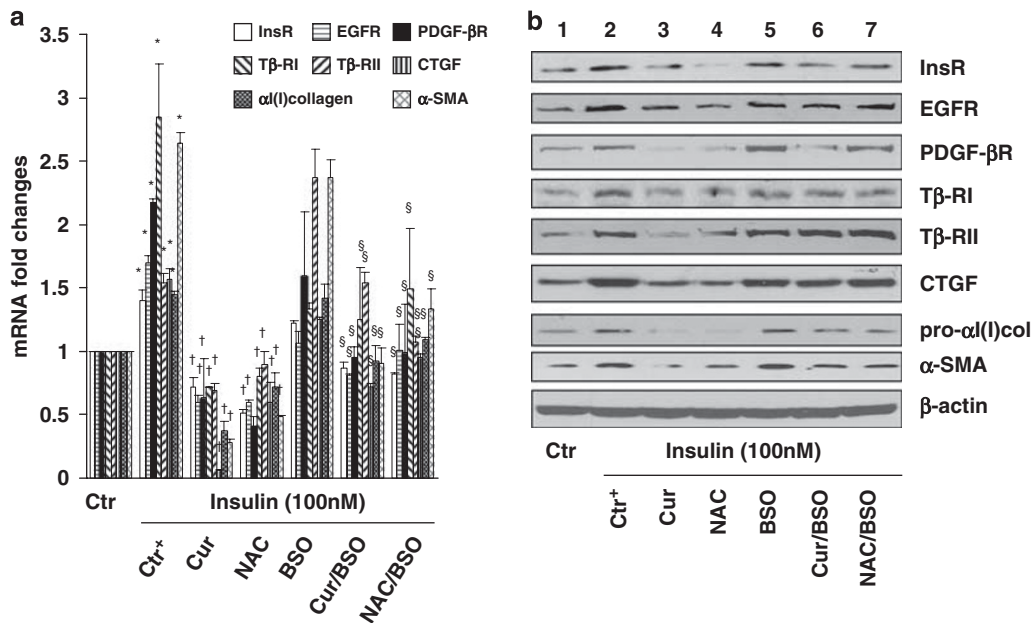
**Figure 8** Curcumin eliminates the effect of insulin and induces gene expression of GCLc and GCLm in activated HSCs *in vitro*. Serum-starved HSCs were stimulated with insulin at indicated concentrations in the absence (**a**, **b**) or presence (**c**, **d**) of curcumin at 0–30  $\mu$ M in serum-free media for 24 h. Total RNA or whole cell extracts were prepared for real-time PCR assays (**a**, **c**) or Western blotting analyses (**b**, **d**).  $\beta$ -actin was used as an invariant internal control for calculating mRNA fold changes ( $n = 3$ ). \* $P < 0.05$ , versus cells with no treatment (the corresponding first column on the left in **a**, **c**); † $P < 0.05$ , versus cells treated with insulin only (the corresponding second column on the left in **a**, **c**).  $\beta$ -actin was used as an internal control for equal loading (**b**, **d**). A representative was shown from three independent experiments.

concentrations in the presence or absence of curcumin at indicated doses in serum-depleted media for 24 h. Total RNA and whole cell extracts were prepared from the cells. Gene expression of GCLc and GCLm were analyzed using real-time PCR and Western blotting analyses. It was observed that insulin reduced the levels of GCLc and GCLm transcripts (Figure 8a) and proteins (Figure 8b). The inhibitory effect of insulin was dose-dependently abrogated by curcumin (Figures 8c and d). These results supported our postulation and showed that curcumin increased the activity of GCL and elevated the level of cellular GSH in insulin-activated HSCs by inducing gene expression of GCLc and GCLm.

#### De Novo Synthesis of GSH is Required for Curcumin to Inhibit Gene Expression of InsR and Other Key Proteins Relevant to HSC Activation Induced by Insulin

To elucidate the mechanisms by which curcumin inhibited InsR gene expression in HSCs, we presumed that the elevation of the level of cellular GSH had a critical role in the curcumin-dependent suppression of expression of InsR gene and other genes relevant to HSC activation, as observed in Figures 1 and 4. To test this presumption, levels of cellular GSH in cultured HSCs were altered by the following well-known manipulators of GSH synthesis. *N*-acetyl-cysteine (NAC) is a precursor of GSH and increases GSH contents by supplying cysteine.<sup>33</sup> *L*-Buthionine sulfoximine (BSO) is a specific inhibitor of GCL, which depletes cellular GSH.<sup>34</sup> We

previously showed that BSO alone caused a time- and dose-dependent reduction in the level of GSH in cultured HSCs.<sup>23</sup> Serum-starved HSCs were divided into two groups. In one group, cells were stimulated with or without insulin (100 nM) and curcumin (20  $\mu$ M) or NAC (5 mM) in serum-free media for 24 h. In another group, cells were pretreated with BSO (0.25 mM) for 1 h before addition of insulin (100 nM) and curcumin (20  $\mu$ M) or NAC (5 mM) in serum-free media for additional 24 h. Total RNA and whole cell extracts were prepared. As shown using real-time PCR (Figure 9a) and Western blotting analyses (Figure 9b), compared with the untreated controls (the corresponding first columns and wells), insulin increased, as expected, the levels of mRNA and proteins of InsR and other key genes relevant to HSC activation (the corresponding second columns and wells), including  $\alpha$ I(I) collagen,  $\alpha$ -SMA and pro-mitogenic PDGF- $\beta$ R and EGFR, as well as pro-fibrogenic T $\beta$ -RI and T $\beta$ -RII and CTGF. NAC (the fourth columns and wells) mimicked the role of curcumin (the third columns and wells) and dramatically eliminated the inhibitory effect of insulin on the expression of InsR and the other key genes relevant to HSC activation. The depletion of intracellular GSH by pretreatment with BSO apparently eliminated the effects of both curcumin (the sixth columns and wells) and NAC (the seventh columns and wells) on the suppression of expression of the genes, including InsR, in activated HSCs *in vitro*. Taken together, these results showed that *de novo*



**Figure 9** De novo synthesis of GSH is required for curcumin to suppress gene expression of InsR and other proteins relevant to HSC activation induced by insulin. Serum-starved HSCs were divided into two groups. One group of cells was treated with or without insulin (100 nM) and curcumin (Cur) (20  $\mu$ M) or NAC (5 mM) in serum-free media for 24 h. Another group of cells was pretreated with BSO (0.25 mM) for 1 hr before addition of insulin (100 nM) and curcumin (20  $\mu$ M) or NAC (5 mM) in serum-free media for additional 24 h. Total RNA or whole cell extracts were respectively prepared for real-time PCR assays (a) or for Western blotting analyses (b).  $\beta$ -actin was used as an invariant internal control for calculating mRNA fold changes ( $n = 3$ ). \* $P < 0.05$  versus cells with no treatment (the first column);  $^{\dagger}P < 0.05$  versus cells treated with insulin only (the second column);  $^{\ddagger}P < 0.05$  versus cells treated with insulin and Cur (the third column) or NAC (the fourth column).  $\beta$ -actin was used as an internal control for equal loading (b). Blots were representatives from three independent Western blotting analyses experiments.

synthesis of GSH was required for curcumin to inhibit gene expression of InsR and other key proteins relevant to HSC activation induced by insulin.

## DISCUSSION

Development of steatosis, steatohepatitis and fibrosis is a common outcome of type II diabetic patients with hyperinsulinemia. A large number of studies have focused on the effect of the elevated levels of insulin on hepatocytes.<sup>2</sup> However, very few studies have addressed the effects of the elevated levels of insulin on activation of HSCs, which are targets and responding cells to pathological insults in the state of hyperinsulinemia.<sup>2</sup> Previous studies suggested that insulin stimulated HSC activation *in vitro* by inducing mitogenesis and collagen synthesis.<sup>12</sup> The underlying mechanisms remain largely to be defined. Curcumin has received attention as a promising dietary component for the protection against fibrogenic insults.<sup>18</sup> The current study was designed to evaluate the effect of curcumin on eliminating the role of insulin in the stimulation of HSC activation and to further elucidate the underlying mechanisms. Our results suggested that curcumin eliminated the role of insulin in stimulating HSC activation. Curcumin interrupted insulin signaling in HSCs by reducing the phosphorylation levels of InsR and downstream inter-mediators and by dramatically suppressing gene expression of InsR. Additional experiments

showed that phytochemical attenuated insulin-induced oxidative stress in HSCs by increasing the activity of GCL and stimulating *de novo* synthesis of GSH. The latter was required for curcumin to suppress expression of InsR and other genes relevant to HSC activation.

Insulin signaling is initiated by the binding of insulin to InsR and subsequently activating downstream signaling cascades. We postulated that InsR might be the first target for curcumin to interrupt insulin signaling. Our results supported the postulation and showed that curcumin exerted its action in two steps. Curcumin eliminated the instant action of insulin by reducing the phosphorylation levels of InsR and its downstream inter-mediators, including ERK, JNK, PI3K and AKT (Figure 3). This action was for a short term. On the other hand, curcumin suppressed gene expression of InsR, leading to the reduction in the bioavailability of InsR to the ligand insulin (Figure 4). This action was slower, but lasted longer, not less than 24 h. Previous studies have shown similar functions of curcumin in interrupting signal transduction pathways for PDGF, EGF and TGF- $\beta$ .<sup>20,28,35</sup> Curcumin reduced the phosphorylation levels of PDGF- $\beta$ R and TGF- $\beta$  receptors, as well as suppressed gene expression of the receptors in activated HSCs *in vitro*. These results suggest that curcumin might be a broad-spectrum inhibitor and non-specifically interrupts signaling pathways involved in the activation of HSCs. The underlying mechanisms remain

elusive. Other studies have indicated the role of GSH in the physiological modulation of protein tyrosine phosphatase 1B, a key enzyme in the downregulation of insulin signaling.<sup>36</sup> We previously observed that it took several hours for curcumin to increase the level of cellular GSH in HSCs,<sup>26</sup> because the process required the transcription and translation of GCL genes. As curcumin rapidly reduced the phosphorylation levels of InsR and the downstream inter-mediators, it is unlikely that the inhibitory effect of curcumin results from its capability to elevate the level of cellular GSH. We recently observed that curcumin instantly and dramatically reduced the level of cellular  $\text{Ca}^{++}$  in cultured HSCs (unpublished observations by Lin and Chen). Accumulating evidence has indicated the role of calcium in regulation of the activities of protein tyrosine kinases and phosphatases.<sup>37–39</sup> Additional experiments are necessary to explore the mechanisms by which curcumin acts as a protein tyrosine kinase inhibitor and/or a phosphatase activator to reduce the phosphorylation levels of InsR and downstream signaling inter-mediators.

A critical question was raised while performing our pilot experiments whether curcumin would interrupt the insulin signaling in other insulin-responding cells, including hepatocytes and muscle cells. If so, curcumin would deteriorate hyperglycemia and diabetes. It is noteworthy that curcumin has shown distinct effects on regulating gene expression depending on cell types.<sup>40</sup> Curcumin induces gene expression of low-density lipoprotein receptor (LDLR) in hepatoma cell line, HepG2,<sup>41</sup> which might result in an increase in the endocytosis of plasma LDL by hepatocytes and a reduction in the level of plasma LDL. However, curcumin suppresses gene expression of LDLR in cultured HSCs,<sup>42</sup> which attenuates the stimulatory role of LDL in the activation of HSCs. Curcumin and its major metabolite increase the number of total cellular insulin-binding sites in circulating erythrocytes in a streptozotocin-induced diabetic rat model.<sup>43</sup> In addition, curcumin significantly reduces blood glucose levels and improves insulin resistance, glucose tolerance as well as lipid profiles in *db/db* mice.<sup>44</sup> Current available results, together with our pilot observations, have suggested that curcumin has anti-hyperglycemic and anti-diabetic effects in diabetic animal models.<sup>44–47</sup> Although beyond the scope of this report, it is of interest to address the underlying mechanisms by which curcumin shows distinct effects on different cell types, including HSCs and hepatocytes.

Curcumin is a potent antioxidant whose antioxidant capacity is 100-fold stronger than vitamin E/C.<sup>17</sup> The underlying mechanisms are largely undefined. NAC, as an antioxidant, has been studied to inhibit hepatic fibrosis in animal models.<sup>48–50</sup> However, results are not always promising and consistent. It is noteworthy that compared with the effects of curcumin at 20  $\mu\text{M}$ , NAC at a much higher concentration (5 mM) showed similar, if not weaker, effects on the attenuation of the insulin-stimulatory effects

(Figure 9). This observation suggested that curcumin, compared with NAC, might act in a unique and more efficient mechanism to exert its antioxidant properties. In general, one molecule of a 'regular' antioxidant, such as vitamin E, *directly* scavenges one or two free radicals, leading to the attenuation of oxidative stress. In this report, we observed that curcumin *indirectly* attenuated oxidative stress by inducing gene expression of GCL, a key rate-limiting enzyme in *de novo* synthesis of GSH. The enhanced GCL activity catalyzes *de novo* synthesis of GSH, resulting in the removal of many molecules of ROS and the reduction of LPO production induced by insulin in HSCs. This unique mechanism explains, at least partially, its potent antioxidant capacity, which might allow curcumin to succeed where other 'regular' antioxidants have failed to inhibit hepatic fibrogenesis. The level of cellular GSH is mainly determined by GSH synthesis (GSH supply) and GSH-consuming (GSH demand). It bears emphasis that our results do not exclude the role of curcumin in the suppression of GSH-consuming to elevate the level of cellular GSH in activated HSCs.

The toxicity of curcumin to cultured HSCs was previously evaluated.<sup>20</sup> On the basis of the results from LDH release assays, Trypan blue exclusion assays and a rapid recovery of cell proliferation after withdrawal of curcumin, it was concluded that curcumin up to 100  $\mu\text{M}$  was not toxic to cultured HSCs. Curcumin at 20  $\mu\text{M}$  was used in most of our experiments in this report. This working concentration of curcumin is in the range used by many other researchers.<sup>51–53</sup> The systemic bioavailability of curcumin is relatively low.<sup>54</sup> Curcumin concentrations in human plasma can reach up to 2  $\mu\text{M}$  after an oral intake of very high amounts of curcumin.<sup>51</sup> Although higher concentrations of curcumin in the blood might be possible, depending on the actual composition of the food,<sup>54</sup> most pharmacokinetic studies in humans point out that only low  $\mu\text{mol}$  levels of curcumin can be found in the blood. It is noteworthy that because the *in vivo* system is multi-factorial, directly extrapolating *in vitro* conditions and results, for example, effective concentrations, to the *in vivo* system might be misleading.

In summary, our results showed that curcumin attenuated the effects of insulin on stimulating HSC activation, at least partially, by interrupting insulin signaling and attenuating oxidative stress. It bears emphasis that our results do not exclude any other mechanism by which curcumin eliminates the effects of insulin on stimulating HSC activation. Our results in this report suggested that curcumin could be an anti-fibrotic candidate in therapeutic treatment of T2DM and NASH-associated hepatic fibrogenesis.

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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