

Post-translational modification of CREB-1 decreases collagen I expression by inhibiting the TGF- β 1 signaling pathway in rat hepatic stellate cells

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Abstract. Liver fibrosis is a reversible wound-healing response to liver damage. Following liver injury, activated hepatic stellate cells (HSCs) cause excessive deposition of collagen and other components of the extracellular matrix, which may eventually impair liver function. Transforming growth factor- β 1 (TGF- β 1) is critical for the development of liver fibrosis, and acts by activating HSCs, stimulating matrix deposition and inducing other profibrotic responses. Certain studies have demonstrated that the transcription factor, cyclic adenosine monophosphate (cAMP)-response element binding protein-1 (CREB-1) is important in the inhibition of fibrosis; however, the underlying mechanism remains to be elucidated. The present study hypothesized that CREB-1 inhibits fibrosis via blocking the TGF- β 1 signaling pathway in HSCs. Treatment with the cAMP-elevating agent, forskolin stimulated CREB-1 phosphorylation and transcription activation in HSCs. In addition, treatment with the histone deacetylase inhibitor, trichostatin A extended CREB-1 phosphorylation. The present study used a pRSV-CREB-1 expression vector to upregulate CREB-1 gene expression. The results indicated that activated CREB-1 reduced TGF- β 1-stimulated expression of collagen I, activated Smad2/3 and upregulated expression of Smad7. In addition, activated CREB-1 attenuated the activation of extracellular signal-regulated kinase 1/2 induced by TGF- β 1, expression of Ras homolog gene family, member A (RhoA) and Rho-associated coiled-coil containing protein kinase 1. Thus, post-translational modification of CREB-1 inhibited the

profibrotic effects of TGF- β 1 in HSCs via Smad-dependent and -independent signaling pathways.

Introduction

Liver fibrosis is a common pathological process occurring as a result of a variety of liver diseases and lesions. Transforming growth factor (TGF)- β 1 is an important promoter of liver fibrosis (1), which activates the differentiation of fibroblasts into myofibroblasts and stimulates the latter to produce a large quantity of matrix proteins (2).

In mammals, the TGF- β superfamily is composed of three homologous genes, which may interact with each other: TGF- β 1, TGF- β 2 and TGF- β 3. Our previous study demonstrated that TGF- β 3 may significantly inhibit the expression of TGF- β 1 and collagen I, the primary protein of the extracellular matrix (3). A further study demonstrated that the TGF- β 3 promoter contains the cyclic adenosine monophosphate (cAMP)-responsive element (CRE) site (4). CRE binding protein 1 (CREB-1), phosphorylated (p) on Ser-133, combines with this particular site and activates the TGF- β 3 promoter, inducing the secretion of TGF- β 3. Therefore, p-CREB-1 is a critical transcription factor in mediating TGF- β 3 auto-induction (4).

Previous studies have indicated that p-CREB-1 has an inhibitory effect on myocardial and pulmonary fibroses (5,6). In addition, p-CREB-1 may have an antifibrotic effect in liver. However, the antifibrotic effect is counteracted by the inactivation of p-CREB-1 through dephosphorylation (7).

Post-translational modifications, including phosphorylation, are the chemical modifications of newly-synthesized polypeptide chains or proteins, which alter their physical and chemical properties and therefore their biological function. Certain studies have demonstrated that acetylation, another post-translational modification, may alter the conformation of a protein and result in a greater biological effect (8). The present study aimed to identify the underlying mechanisms involved in post-translational modifications of CREB-1 in inhibiting TGF- β 1-mediated liver fibrosis. The results indicated a theoretical basis for clarifying the underlying CREB-1 antifibrotic mechanisms, and thereby provide a novel therapeutic target for the treatment of liver fibrosis.

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Materials and methods

Cell culture and reagents. HSC-T6 rat hepatic stellate cells (HSCs) were provided by the Division of Gastroenterology Laboratory of Union Hospital (Wuhan, China). HSCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO₂. All experiments were conducted when cells were at an exponential stage of growth. Human recombinant TGF- β 1 was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA) and used at a concentration of 10 ng/ml. The cAMP-elevating agent, forskolin (FSK) was purchased from Beyotime Institute of Biotechnology (Haimen, China) and the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) was obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

Transfection and treatment. HSCs were seeded into 6-well plates at a concentration of 2×10^5 /well. Transfection with pRSV-CREB-1 expression vector or an empty vector (Invitrogen; Thermo Fisher Scientific, Inc.) was conducted when cells reached 70–90% confluence. Lipofectamine™ 2000 (10 μ l; Invitrogen; Thermo Fisher Scientific, Inc.) in 250 μ l Opti-minimal essential medium (Opti-MEM; Gibco; Thermo Fisher Scientific, Inc.) was mixed with 4 μ g vector in 250 μ l Opti-MEM and incubated for 20 min at room temperature to allow formation of the transfection complex, which was then added to each well (500 μ l transfection complex in 1,500 μ l DMEM). A total of 6 h following transfection, culture media were replaced with fresh DMEM, and cells were incubated for an additional 18 h. TGF- β 1, FSK or TSA were subsequently added into different wells, to a final concentration of 10 ng/ml, 10 μ M and 100 nM respectively. Treated HSCs were incubated for various times and harvested for subsequent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.). The purity and concentration of the extracted RNA was detected by an ultraviolet spectrophotometer (Eppendorf Biophotometer Plus; Thermo Fisher Scientific, Inc.), and reverse-transcribed to cDNA using PrimeScript™ RTase kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. qPCR was performed in a total volume of 10 μ l with 5 μ l SYBR Green PCR Master mix (Takara Biotechnology Co., Ltd.), 0.5 μ l of each primer (0.2 μ mol in total), 1 μ l cDNA (1:10 dilution) and 3 μ l double-distilled water. Primer sequences are presented in Table I. All cDNA samples were tested in duplicate using a StepOne™ PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), with thermocycling conditions as follows: An initial denaturation step of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. Fluorescence data from each sample was analyzed using the 2^{−ΔΔC_q} method (9), with GAPDH gene expression serving as an endogenous control.

Extraction of nuclear protein. Nuclear proteins from HSCs cultured in 100-mm culture dishes were extracted using a

Nuclear-Cytosol Extraction kit (Applygen Technologies, Inc., Beijing, China). Cells were washed with cold PBS three times, lysed in 300 μ l cytoplasm extraction buffer A for 10 min and 20–30 μ l cytoplasm extraction buffer B for 1 min. Following a 5 min centrifugation at 1,000 \times g, the pellet containing nuclei was collected and resuspended in 70–100 μ l nuclear extraction buffer for 30 min. The solution was centrifuged at 12,000 \times g for 10 min, and the supernatant containing nuclear proteins was collected and stored at −70°C until analysis. All procedures were carried out at 4°C. Protein concentrations were determined using a Bicinchoninic Acid Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.).

Western blotting. Whole-cell lysates were obtained using radioimmunoprecipitation buffer (Applygen Technologies, Inc.) containing protein phosphatase inhibitor (Applygen Technologies, Inc.), according to the manufacturers' protocol; the nuclear extracts were obtained as described above. Equal quantities of protein (30–50 μ g) from the lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature, and incubated individually with the following primary antibodies: Rabbit anti-CREB-1 (1:1,000; catalog no. 9197; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-mothers against decapentaplegic (Smad) 3 (1:1,500; catalog no. 1735-1; Epitomics, Burlingame, CA, USA), rabbit anti-extracellular signal-regulated kinase 1/2 (ERK1/2; 1:1,000; catalog no. 4695; Cell Signaling Technology, Inc.), rabbit anti-collagen I (1:5,000; catalog no. ab34710; Abcam, Cambridge, UK) and rabbit anti-GAPDH (1:1,000; catalog no. GTX100118; GeneTex, Inc., Irvine, CA, USA), overnight at 4°C. Separate membranes were blocked with 5% bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA) in TBST for 1 h at room temperature, and incubated with rabbit anti-p-CREB (Ser-133; 1:1,000; catalog no. 9198; Cell Signaling Technology, Inc.), goat anti-p-Smad2/3 (Ser-423/425; 1:100; catalog no. sc-11769; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit anti-p-ERK1/2 (Thr-202/Tyr-204; 1:1,000; catalog no. 4370S; Cell Signaling Technology, Inc.). Following three washes in TBST, membranes were incubated with anti-rabbit or anti-goat IgG horseradish peroxidase-conjugated secondary antibodies (1:8,000; catalog nos. 111-035-003 and 305-035-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. Target proteins were detected using an Enhanced Chemiluminescence Detection kit (Thermo Fisher Scientific, Inc.). Densitometric analysis was performed using AlphaView Stand Alone software version 3.4.0 (ProteinSimple, San Jose, CA, USA).

Statistical analysis. All experiments were performed three times independently. Data were analyzed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA), using unpaired Student's *t*-tests and one-way analyses of variance followed by Fisher's least significant difference post-hoc test. Data are presented as the mean \pm standard deviation. *P* < 0.05 was considered to indicate a statistically significant difference. Graphs were produced using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'-3')	
	Sense	Antisense
CREB-1	CAGTGCCAACCCCGATTTA	TTGCTCCTCCCTGGGTAATG
Collagen I	TTGTGCGATGACGTGATCTGT	TTGGTCGGTGGGTGACTCTG
Smad3	GGGCCTGCTGTCCAATGT	AATGTGCCGCCTTGTAAGCT
Smad7	TGGATGGCGTGTGGGTTTA	TGGCGGACTTGATGAAGATG
ERK1/2	TTTGGTCTGTGGGCTGCAT	TCCTGGGAAGATAGGCCTGTT
RhoA	TCGGAATGATGAGCACACAAG	GGTTTTACCGGCTCCTGCTT
ROCK1	TTCATTCTACCTCTACCACT	GGCTTAAAAACATGCCACAA
GAPDH	GTATGACTCTACCCACGGCAAGT	TTCCCGTTGATGACCAGCTT

CREB-1, cyclic adenosine monophosphate response element binding protein-1; Smad, mothers against decapentaplegic; ERK1/2, extracellular signal-regulated kinase 1/2; RhoA, Ras homolog gene family member A; ROCK1, Rho-associated coiled-coil containing protein kinase 1.

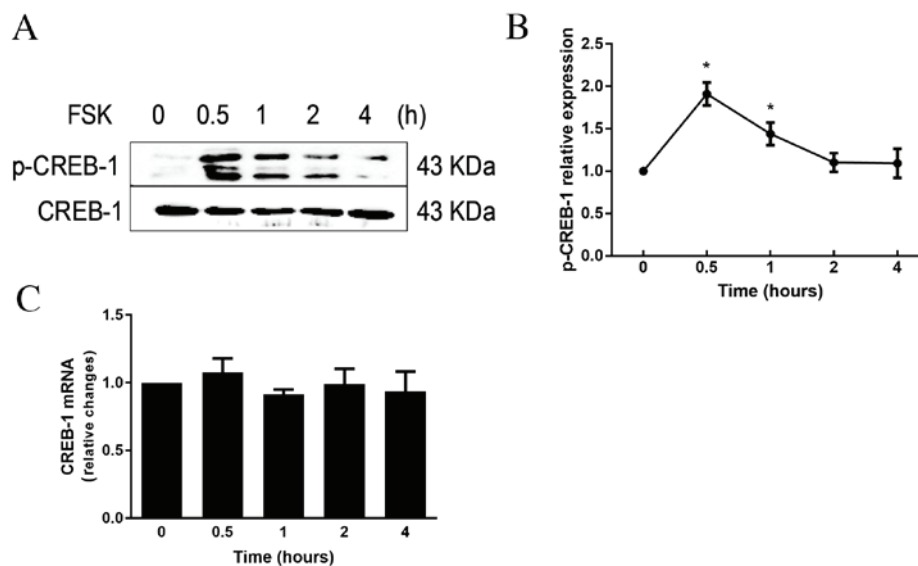


Figure 1. Time-dependent CREB activation by FSK. HSCs were stimulated with 10 μ M FSK for various times, nuclear proteins were harvested and (A) protein expression of p-CREB-1 and CREB-1 were detected by western blotting. (B) Quantification of western blotting revealed that FSK treatment increased protein expression levels of p-CREB-1 in a time-dependent manner. (C) CREB-1 mRNA was detected by reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH expression. There were no statistically significant differences between the groups at any time points. Data are expressed as the mean \pm standard deviation of at least three independent experiments. * $P < 0.05$ vs. 0 h group. CREB-1, cyclic adenosine monophosphate-response element binding protein-1; FSK, forskolin; HSCs, hepatic stellate cells; p, phosphorylated.

Results

cAMP-elevating agent FSK activates CREB-1. HSCs were stimulated with the cAMP-elevating agent, FSK for various times. The protein expression levels of CREB-1 remained unaltered regardless of the duration of stimulation (Fig. 1A). However, phosphorylation levels of CREB-1 at Ser-133 peaked at 30 min (1.9-fold greater than control group; $P = 0.011$), then gradually declined, and maintained a relatively stable level after 1 h (Fig. 1A and B), demonstrating inconsistencies with a previous study on cardiac fibroblasts (10). In addition, CREB-1 mRNA expression levels following FSK stimulation in HSCs were examined. There were no statistically

significant differences between the groups at any time points ($P = 0.863$; Fig. 1C). These results indicate that CREB-1 is activated by FSK in a time-dependent manner.

CREB-1 decreases collagen I expression in HSCs. A pRSV-CREB-1 expression vector and an empty vector were used to transfect HSCs, to investigate whether CREB-1 inhibited collagen I expression. Transfection with pRSV-CREB-1 expression vector increased CREB-1 mRNA expression levels (161.3-fold greater than the empty vector group; $P < 0.001$; Fig. 2A), which was accompanied by a substantial increase in CREB-1 protein expression levels (2.4-fold greater than the empty vector group; $P = 0.003$; Fig. 2B and C) and a slight

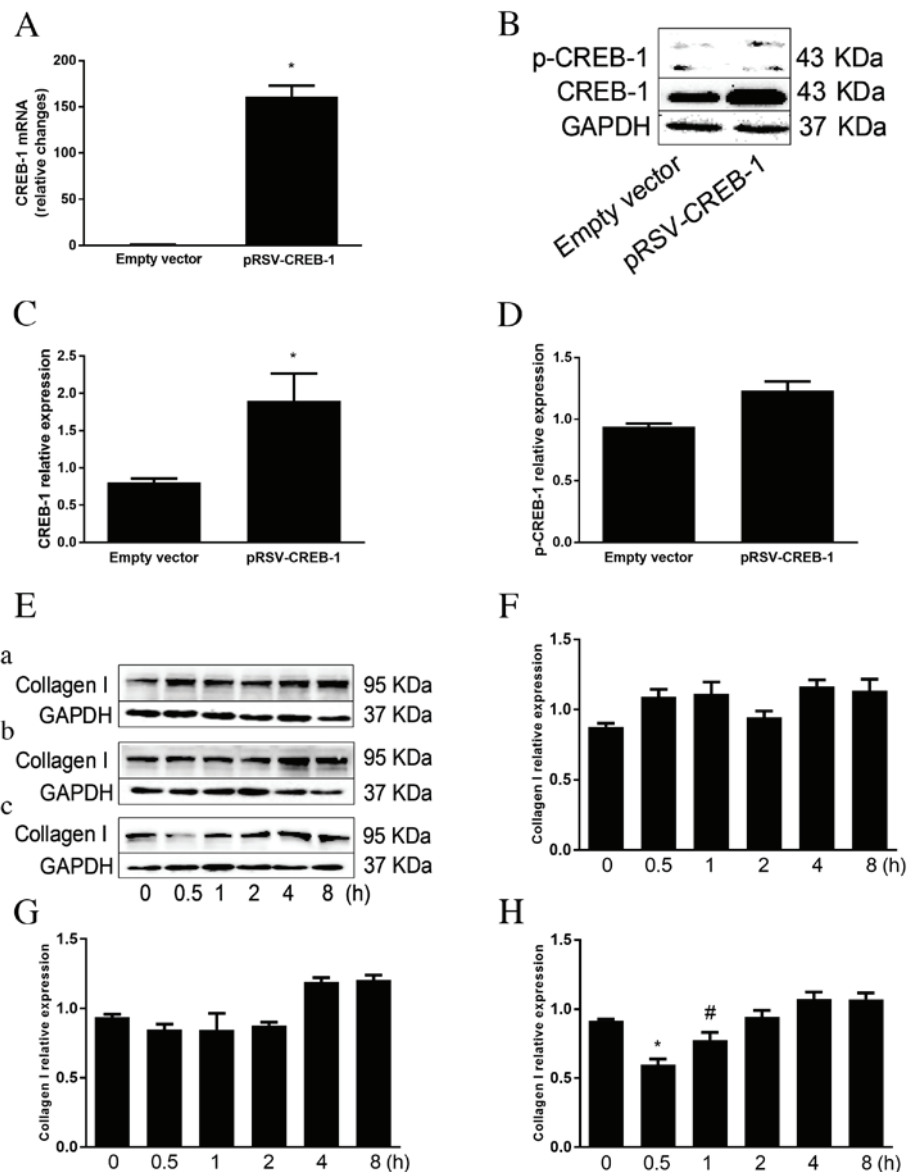


Figure 2. CREB-1 decreases collagen I expression in HSCs. (A-D) HSCs were transfected with pRSV-CREB-1 expression vector or empty vector. A total of 24 h after transfection (A) CREB-1 mRNA was detected by reverse transcription-quantitative polymerase chain reaction and normalized to GAPDH expression, and (B and C) CREB-1 and (D) p-CREB-1 nuclear protein expression levels were detected by western blotting. Transfection with pRSV-CREB-1 expression vector increased CREB-1 mRNA and protein expression levels, and slightly increased p-CREB-1 protein expression levels, compared with the empty vector control. (E) HSCs were transfected with (a) empty vector, (b) pRSV-CREB-1 expression vector or (c) pRSV-CREB-1 expression vector, followed by treatment with 10 μ M FSK 24 h after transfection. Total protein was extracted and collagen I was detected by western blotting. Quantification of (F) empty vector, (G) pRSV-CREB-1 expression vector and (H) pRSV-CREB-1 expression vector followed by FSK treatment. Activated CREB-1 significantly reduced the expression of collagen I with a peak at 0.5 h. The negative effect of CREB-1 on collagen I protein expression levels gradually decreased or was lost over time. Data are presented as the mean \pm standard deviation of at least three independent experiments. (A-D) * P <0.01 vs. empty vector group; (E-H) # P <0.05 and * P <0.01 vs. 0 h group. CREB-1, cyclic adenosine monophosphate-response element binding protein-1; HSCs, hepatic stellate cells; p, phosphorylated; FSK, forskolin.

increase in p-CREB-1 protein expression ($P=0.056$; Fig. 2D). HSCs were treated with FSK 24 h following pRSV-CREB-1 transfection. Activated CREB-1 significantly reduced the expression of collagen I with a peak at 0.5 h (1.5-fold lower than the empty vector group; $P=0.003$), consistent with the peak of CREB-1 activation. However, the negative effect of CREB-1 on collagen I protein expression levels gradually decreased or was lost over time (Fig. 2E-H).

CREB-1 decreases collagen I expression via inhibiting the Smad-dependent TGF- β 1 signaling pathway. To elucidate

the underlying mechanism involved in CREB-1 inhibition of TGF- β 1-mediated liver fibrosis, TGF- β 1 and/or FSK were used to treat transfected cells. As presented in Fig. 3A and B, TGF- β 1 treatment significantly increased collagen I protein expression levels in the empty vector group ($P=0.025$); however, the protein expression levels of collagen I demonstrated only a slight increase under TGF- β 1 stimulation in the pRSV-CREB-1 expression vector group. In addition, the collagen I expression level was reduced in each pRSV-CREB-1 group compared with the corresponding empty vector group. The proteins of the Smad family were the first identified substrates of type I

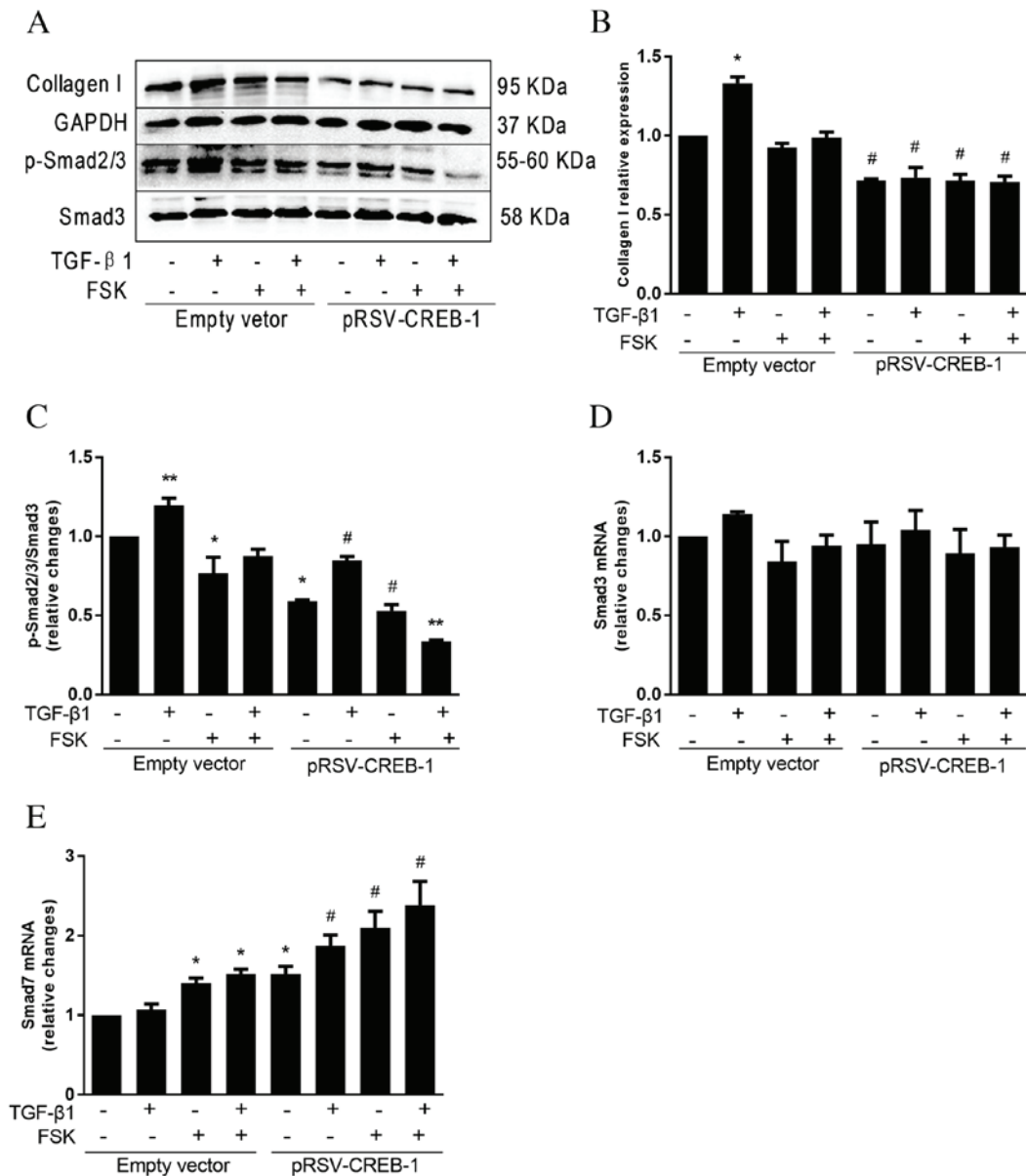


Figure 3. CREB-1 inhibits the TGF- β 1 signaling pathway via Smad-dependent pathways. Cells were transfected with pRSV-CREB-1 expression vector or empty vector, and 24 h later were treated with 10 μ M FSK and/or 10 ng/ml TGF- β 1 for 1 h. (A) Whole-cell lysates were analyzed by western blotting using antibodies against collagen I, p-Smad2/3 (Ser-423/425), Smad3 or GAPDH. (B) TGF- β 1 significantly increased collagen I protein expression levels in the empty vector group, but not in the pRSV-CREB-1 expression vector group. (C) p-Smad2/3 protein expression levels were consistent with collagen I; however, p-Smad2/3 expression was suppressed in the FSK plus TGF- β 1-treated group following CREB-1 overexpression. Total RNA was collected and Smad3 and Smad7 mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction. (D) No differences were observed in Smad3 mRNA expression levels between groups. (E) Smad7 mRNA levels were increased following transfection with the pRSV-CREB-1 expression vector, compared with the empty vector control. mRNA results were normalized to GAPDH expression. Data are expressed as the mean \pm standard deviation of at least three independent experiments. * P <0.05 vs. no treatment group; ** P <0.05 vs. all other groups; # P <0.05 vs. corresponding empty vector control group. CREB-1, cyclic adenosine monophosphate-response element binding protein-1; TGF- β 1, transforming growth factor- β 1; FSK, forskolin; Smad, mothers against decapentaplegic; p, phosphorylated.

receptor kinases and are key in the TGF- β 1 signaling pathway. p-Smad2/3 is considered the final phosphorylation step and the component of the p-Smad complex that translocates to the nucleus to regulate target genes. p-Smad2/3 protein expression levels were consistent with collagen I; however, p-Smad2/3 expression was suppressed significantly in the FSK plus TGF- β 1-treated group following CREB-1 overexpression (P <0.001; Fig. 3C). No differences were observed in Smad3

mRNA expression levels between groups (P =0.177; Fig. 3D). The antagonistic Smad7 acts in opposition to p-Smads (11). In the present study, Smad7 mRNA expression levels were greater in the FSK-treated group compared with the TGF- β 1-treated group (Fig. 3E). In addition, Smad7 mRNA expression levels were greater in each pRSV-CREB-1 group, compared with the respective empty vector control, particularly in the FSK plus TGF- β 1-treated group (P <0.001).

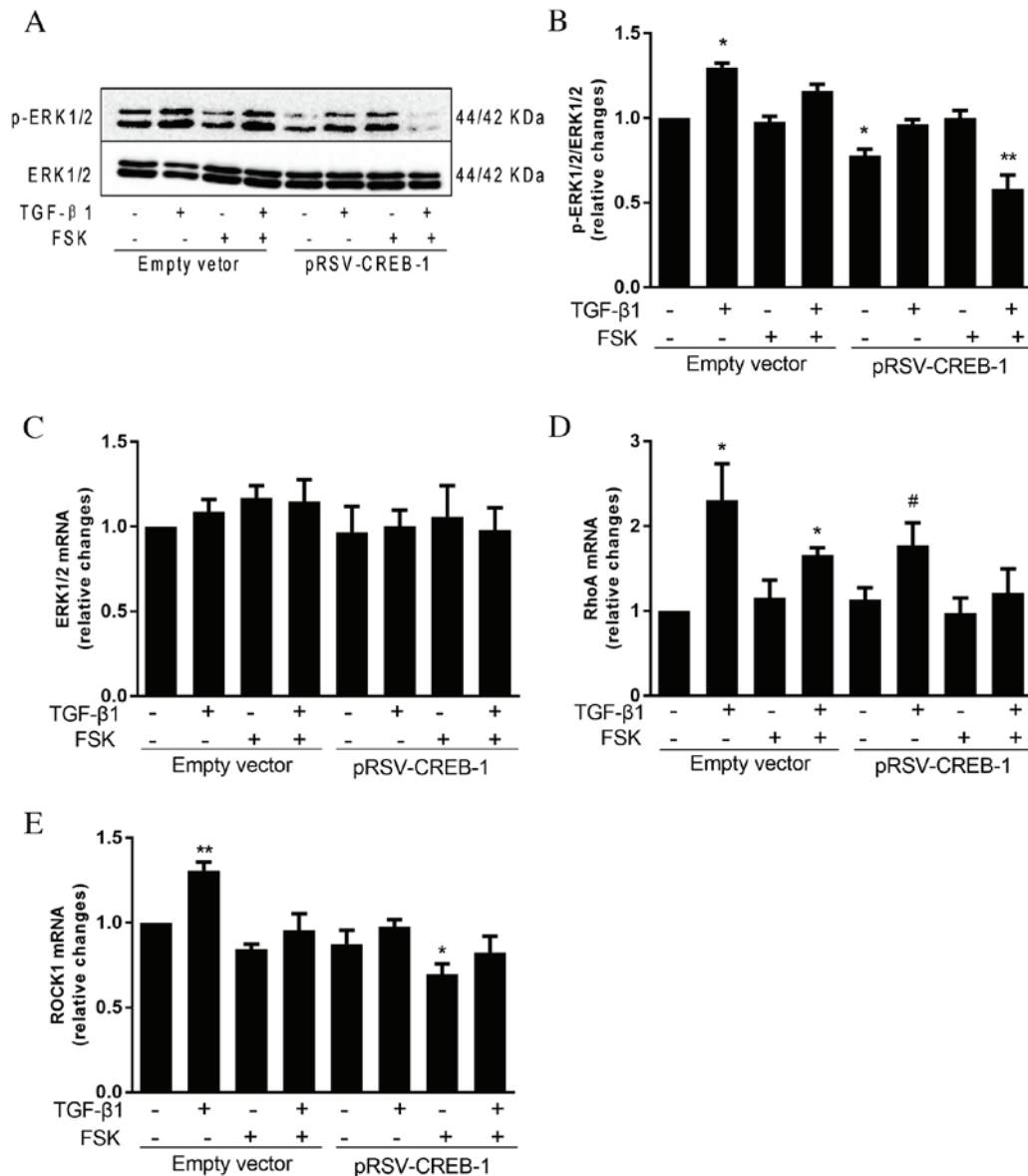


Figure 4. CREB-1 inhibits the TGF- β 1 signaling pathway via Smad-independent pathways. Cells were transfected with a pRSV-CREB-1 expression vector or an empty vector and 24 h later treated with 10 μ M FSK and/or 10 ng/ml TGF- β 1 for 1 h. (A) Whole-cell lysates were analyzed by western blotting using antibodies against p-ERK1/2 (Thr-202/Tyr-204) and ERK1/2. (B) Quantification of western blotting revealed that CREB-1 overexpression significantly attenuated the activation of ERK1/2 induced by TGF- β 1. The total RNA was collected, and (C) ERK1/2, (D) RhoA and (E) ROCK1 mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction. TGF- β 1 stimulated the expression of RhoA and ROCK1; however, CREB-1 overexpression weakened the effect of TGF- β 1 stimulation. mRNA results were normalized to GAPDH expression. Data are expressed as the mean \pm standard deviation of at least three independent experiments. * P <0.05 vs. no treatment group; ** P <0.05 vs. all other groups; # P <0.05 vs. corresponding empty vector control group. ERK1/2, extracellular signal-regulated kinase 1/2; CREB-1, cyclic adenosine monophosphate response element binding protein-1; FSK, forskolin; TGF- β 1, transforming growth factor- β 1; p, phosphorylated; RhoA, Ras homolog gene family member A; ROCK1, Rho-associated coiled-coil containing protein kinase 1.

CREB-1 decreases collagen I expression via inhibition of the Smad-independent TGF- β 1 signaling pathway. p-ERK1/2 and Ras homolog gene family member A (RhoA)/Rho-associated coiled-coil containing protein kinase 1 (ROCK1) signaling are involved in the TGF- β 1 signaling pathway in cardiac fibroblasts and corneal myofibroblasts (5,12). These studies indicated that increased cAMP markedly blocks TGF- β 1-mediated activation of collagen synthesis and α -smooth muscle actin expression via inhibition of ERK1/2 phosphorylation and RhoA activation.

The present study hypothesized that activated CREB-1 has a similar role in HSCs. To verify this, p-ERK1/2 and ERK1/2 protein expression levels were analyzed by western blotting, and ERK1/2, RhoA and ROCK1 mRNA expression levels were analyzed via RT-qPCR following CREB-1 overexpression. The results demonstrated inconsistencies with collagen I and p-Smad2/3 data, as p-ERK1/2 levels were slightly, but not significantly, increased by FSK stimulation alone following CREB-1 overexpression ($P=0.201$). However, activated

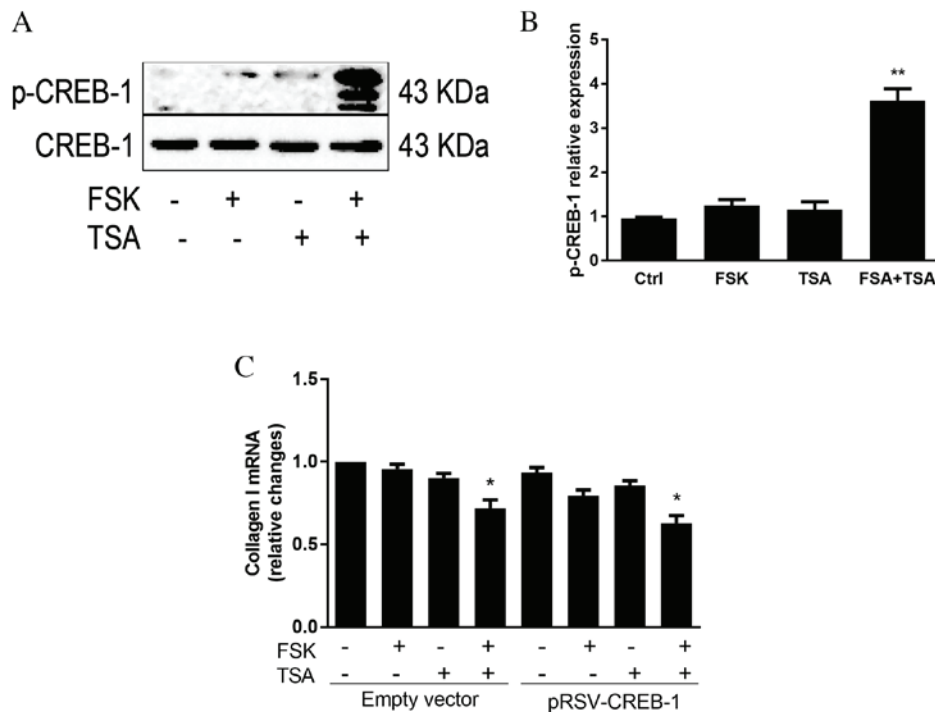


Figure 5. Acetylation extends the function of phosphorylated CREB-1 in decreasing collagen I expression. Cells were stimulated with 10 μ M FSK and/or 100 nM TSA for 4 h following transfection. (A) Nuclear proteins were harvested, and protein expression levels of p-CREB-1 and CREB-1 were detected by western blotting. (B) Quantification of western blotting revealed that in the FSK plus TSA intervention group, expression of p-CREB-1 was significantly increased compared with the control group. (C) Collagen I mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction. Collagen I mRNA expression levels were significantly reduced by 37% following pRSV-CREB-1 transfection in the FSK plus TSA group compared with the no treatment group. mRNA results were normalized to GAPDH expression. Data are expressed as the mean \pm standard deviation of at least three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. no treatment group. CREB-1, cyclic adenosine monophosphate response element binding protein-1; FSK, forskolin; p, phosphorylated; TSA, trichostatin A.

CREB-1 significantly attenuated activation of ERK1/2 induced by TGF- β 1 ($P < 0.001$; Fig. 4A and B). No statistically significant differences were observed in ERK1/2 mRNA expression levels ($P = 0.453$; Fig. 4C). TGF- β 1 stimulated the expression of RhoA (Fig. 4D) and ROCK1 (Fig. 4E); however, overexpression of CREB-1 reduced RhoA and ROCK1 mRNA expression levels, weakening the effect of TGF- β 1 stimulation.

Acetylation extends the function of phosphorylated CREB-1 in decreasing expression levels of collagen I. The above results indicated that CREB-1 may be activated by FSK, and have a key antifibrotic role. However, inactivated p-CREB-1 diminishes the effect of FSK stimulation. A previous study revealed that the HDAC inhibitor TSA acetylates CREB-1 and extends its phosphorylation level in an NIH/3T3 D5 cell line (13). To determine whether TSA has the same effect in HSCs, cells were stimulated with FSK and/or TSA to induce CREB-1 post-translational modification. The results demonstrated that in the FSK plus TSA group, protein expression levels of p-CREB-1 were significantly increased compared with the control group (3.6-fold greater than control group; $P < 0.001$; Fig. 5A and B). Differences in CREB-1 expression between the other groups was not statistically significant ($P = 0.139$). In addition, following 4 h of treatment with FSK and TSA in the pRSV-CREB-1 group, collagen I mRNA expression levels were significantly reduced by 37% compared with the no treatment group ($P = 0.011$; Fig. 5C).

Discussion

As an important member of the activating transcription factors/CREB family, CREB-1 is involved in a variety of biological functions, including inhibiting inflammation and apoptosis, enhancing immunity and memory, and regulating growth and development (12,14). Previous studies have revealed that CREB-1 has antifibrotic effects in lung, myocardial and corneal tissue (5,6,15). However, the role of CREB-1 in liver fibrosis remains to be elucidated. The results of the present study demonstrated that CREB-1 activated by FSK significantly reduces collagen I expression in HSCs. This suggested that CREB-1 exerts an antifibrotic effect in liver tissue.

TGF- β 1 has an important role in the liver fibrotic response, and inhibition of the TGF- β 1 signaling pathway is a potential therapeutic strategy for the treatment of fibrosis. TGF- β 1 binds to TGF- β 1 receptors, which induce the phosphorylation of Smad2. Smad2 in turn phosphorylates the highly analogous protein, Smad3, referred to as R-Smad, and induces the recruitment of Smad4. The activated Smad3/4 complex translocates into the nucleus to interact with Smad-binding elements and regulate downstream genes, particularly collagen (16). By contrast, Smad7 acts in opposition to this process by binding to the type I receptor and preventing recruitment of phosphorylated Smad2 (17). TGF- β 1 activates Smad proteins, ERK1/2 and RhoA/ROCK1 (11,18,19). In the present study, TGF- β 1 induced

the phosphorylation of Smad2/3 in HSCs, as well as the phosphorylation of ERK1/2 and the expression of RhoA/ROCK1. This effect was significantly reduced following treatment with FSK. In addition, activation of CREB-1 enhanced expression of the inhibitory factor, Smad7. Notably, the combined treatment of TGF- β 1 and FSK in CREB-1-transfected HSCs resulted in a greater reduction in p-Smad2/3 and p-ERK1/2 expression levels and a greater increase in Smad7 expression levels compared with FSK treatment alone. These results suggested that activated CREB-1 has a strong inhibitory effect on TGF- β 1-mediated activation during fibrotic responses via blocking Smad-dependent and -independent signaling pathways. However, other signaling pathways, including phosphatidylinositol-3-kinase/serine-threonine kinase, c-Jun N-terminal kinase, mitogen-activated protein kinase and TGF- β -activated kinase 1 have been reported to be associated with fibrosis (20-23). The specific mechanism underlying CREB-1 regulation in HSCs remains to be fully elucidated.

Post-translational modifications of CREB-1 include phosphorylation, acetylation, ubiquitination, sumoylation and glycosylation (8). Phosphorylation has an important role in transcriptional activation and biological functions. Without phosphorylation, CREB-1 has almost no biological activity. However, phosphorylated CREB-1 may be hydrolyzed in internal environments. A previous study revealed that acetylation may improve the stability of phosphorylated proteins by altering the space conformation of CREB-1 and allowing its escape from hydrolysis (13). Results from the present study demonstrated that p-CREB-1 reached a peak level following 30 min of FSK treatment, then declined gradually. The previous study on cardiac fibroblasts demonstrated that the peak level was reached at 5 min and then declined rapidly (10). This may have been due to the experimental conditions, or the fact that the former was a cell line, whereas the latter was a primary cell (10). HSCs treated with cAMP-elevating agent or cAMP-elevating agent plus HDAC inhibitor for 4 h demonstrated a significant increase in the expression of Ser-133-phosphorylated CREB compared with FSK treatment alone. Furthermore, co-treatment reduced collagen I mRNA expression levels significantly in the CREB-1 overexpression group. These results suggested that a HDAC inhibitor may prolong p-CREB-1 phosphorylation at Ser-133 and enhance its antifibrotic capacity.

CREB-binding protein (CBP) was originally identified as a co-activator of CREB. CBP acts as a bridging factor between CREB and transcription factor IIB. CBP, containing its homologue p300, possesses intrinsic histone acetyltransferase activity; CBP/p300 acetylates histones and non-histone proteins, including transcription factors (24). The phosphorylation of CREB at Ser-133 leads to the association of CREB via the kinase-inducible domain with a short domain in CBP termed the kinase-inducible domain interacting domain; p-CREB is further acetylated, and CREB activity during the late attenuation phase is prolonged (25). However, CBP, as a broad-spectrum coactivator, directly interacts with R-Smad-Smad4 via the C-terminal domain to activate the transcription of TGF- β 1-responsive genes (26). Therefore, CREB-1 may compete with R-Smad-Smad4 to interact with CBP. In the present study, the expression of collagen I was significantly reduced by FSK and TSA treatment following

CREB-1 overexpression, demonstrating that excessive acetylated p-CREB-1 may inhibit R-Smad-Smad4 recruitment of CBP, thus inhibiting TGF- β 1 signaling pathways. However, additional studies including *in vivo* experiments are required to provide further evidence for the antifibrotic effect of acetylated p-CREB-1.

In conclusion, the results of the present study demonstrated that p-CREB-1 inhibits TGF- β 1-mediated liver fibrosis by blocking the Smad-dependent and -independent signaling pathways in HSCs. Furthermore, the results of the current study revealed that CREB-1 is not sustainably activated; however, acetylation extends CREB-1 phosphorylation and enhances its antifibrotic effect. The results of the present study suggested that CREB-1 may be a useful potential therapeutic target for the treatment of liver fibrosis.

Acknowledgements

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