

# Caffeic acid phenethyl ester attenuates pro-inflammatory and fibrogenic phenotypes of LPS-stimulated hepatic stellate cells through the inhibition of NF- $\kappa$ B signaling

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**Abstract.** Hepatic stellate cells (HSCs) are the major cell type involved in liver fibrosis. Lipopolysaccharide (LPS)-mediated signaling through Toll-like receptor 4 (TLR4) in HSCs has been identified as a key event in liver fibrosis, and as the molecular link between inflammation and liver fibrosis. In this study, we investigated the effects of caffeic acid phenethyl ester (CAPE), one of the main medicinal components of propolis, on the pro-inflammatory and fibrogenic phenotypes of LPS-stimulated HSCs. HSCs from rats were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM). Following treatment with LPS, HSCs showed a strong pro-inflammatory phenotype with an upregulation of pro-inflammatory mediators, and a fibrogenic phenotype with enhanced collagen synthesis, mediated by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). CAPE significantly and dose-dependently reduced LPS-induced nitrite production, as well as the transcription and protein synthesis of monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS), as determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), western blotting and enzyme-linked immunosorbent assays (ELISA). CAPE further reduced the TGF- $\beta$ 1-induced transcription and translation (protein synthesis) of the gene coding for collagen type I  $\alpha$ 1 (col1A1), in LPS-stimulated HSCs. Following LPS stimulation, the phosphorylation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) inhibitor I $\kappa$ B $\alpha$  and consequently, the nuclear translocation of NF- $\kappa$ B, were markedly increased in the HSCs, and these changes were reversed by pre-treatment with CAPE. In conclusion, CAPE attenuates the pro-inflammatory phenotype of LPS-stimulated HSCs, as well as the LPS-induced sensitization of HSCs to fibrogenic cytokines by inhibiting NF- $\kappa$ B signaling. Our results provide new insight into the

treatment of hepatic fibrosis through regulation of the TLR4 signaling pathway.

## Introduction

Liver fibrosis is a wound-healing response to various chronic liver injuries caused by alcoholism, persistent viral and parasite infections, or hereditary metal overload (1,2). Hepatic stellate cells (HSCs) play a pivotal role in liver fibrogenesis. During liver injury, quiescent HSCs transdifferentiate into activated HSCs, also termed myofibroblast (MFB)-like cells. This cell type provides the main components of the extracellular fibrotic matrix, and also produces an array of pro-inflammatory cytokines and chemokines involved in the development of liver fibrosis (3,4). Thus, HSCs are considered the most important target for therapeutic intervention to prevent the development of hepatic fibrosis (1,5).

There is accumulating evidence that the lipopolysaccharide (LPS)/Toll-like receptor 4 (TLR4) signaling pathway in HSCs plays a key role in liver fibrogenesis (6,7). Previous studies have demonstrated that the levels of the LPS endotoxin are elevated in experimental models of hepatic fibrosis (6) and in patients with liver cirrhosis (8,9), and that the administration of antibiotics that reduce the prevalence of LPS can inhibit fibrogenesis and TLR4 expression in the liver (10). In chronic liver diseases, upon the disruption of the intestinal barrier function, the increase in intestinal permeability leads to the translocation of intestine-derived bacterial products to the liver via the portal vein (10,11). Although Kupffer cells are considered to be the main targets of bacterial products in the liver, there is also evidence that HSCs, and not Kupffer cells, are the primary targets of TLR4 ligands, through which they induce a fibrogenic phenotype (6). Both quiescent and activated HSCs express TLR4 and are thereby highly responsive to even low concentrations of LPS. TLR4 signaling was identified as the molecular link between inflammation and hepatic fibrosis (6,12,13). LPS activates the TLR4/myeloid differentiation 2 (MD2) signaling complex through binding to serum and cell surface proteins, including LPS-binding protein (LBP) and CD14. This subsequently activates downstream effectors such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) (14). Finally, these pathways

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regulate the expression of pro-inflammatory cytokines and genes that control cell survival and apoptosis (14).

LPS/TLR4 signaling in HSCs is essential for the development of liver fibrosis. Mice with mutations in the genes coding for TLR4, CD14, LBP and myeloid differentiation factor 88 show reduced liver fibrosis after bile duct ligation (BDL) or treatment with carbon tetrachloride (6,10,15). Seki *et al* (6) analyzed the cell-specific molecular mechanism underlying the role of LPS/TLR4 signaling on liver fibrosis. They demonstrated that chimeric mice that carry TLR4-mutant Kupffer cells and TLR4-intact HSCs develop pronounced fibrosis, while mice that carry TLR4-intact Kupffer cells and TLR4-mutant HSCs develop minimal fibrosis after BDL, indicating that TLR4 is crucial for the induction of hepatic fibrosis in HSCs, but not in Kupffer cells. LPS/TLR4 signaling in HSCs induces the production of chemokines, which recruit Kupffer cells at HSC sites and allow unrestricted activation of HSCs by the Kupffer cell-derived protein known as transforming growth factor- $\beta$  (TGF- $\beta$ ) (5,6,13). Thus, inhibition of LPS/TLR4 signaling in HSCs appears to be a promising strategy for the prevention of liver fibrosis.

Caffeic acid phenethyl ester (CAPE) (Fig. 1) is one of the main medicinal components of propolis, which is a naturopathic medicine collected by honeybees from buds and exudates of conifer trees and plants (16,17). A number of important biological activities have been reported for CAPE, such as antioxidant (18,19), anti-inflammatory (20) and anticancer activities (21). Previous studies have indicated that CAPE is a potent inhibitor of NF- $\kappa$ B (20,22,23). For example, CAPE inhibits *Helicobacter pylori*-induced NF- $\kappa$ B and AP-1 DNA binding activities in a dose and time-dependent manner in gastric epithelial cells (24). In another study conducted on the colorectal carcinoma cell line, HCT116, CAPE affected tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-dependent I $\kappa$ B $\alpha$  degradation and the subsequent nuclear accumulation of NF- $\kappa$ B (p65) through the direct inhibition of the inhibitory protein  $\kappa$ B kinase (IKK), as well as through the activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway (22).

Since LPS/TLR4 signaling plays a crucial role in liver fibrosis in HSCs, the aim of this study was to investigate the effects of CAPE on the pro-inflammatory and fibrogenic phenotypes of LPS-stimulated HSCs.

## Materials and methods

**Animals and reagents.** Male Wistar rats, weighing 450-500 g, were used in the experiments, and were provided by the Laboratory Animal Center of Kunming Medical University. The study was performed in accordance with the principles for the care and use of laboratory animals approved by The Research Ethics Committee of the Kunming General Hospital of PLA (no. K2010-008).

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Los Angeles, CA, USA). CAPE, LPS, TGF- $\beta$ 1, Necodenz, proteinase E and collagenase II were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol, M-MuLV reverse transcriptase, Platinum SYBR-Green qPCR SuperMix-UDG, and enzyme-linked immunosorbent assay (ELISA) kits for assaying rat interleukin-6 (IL-6) and monocyte chemoat-

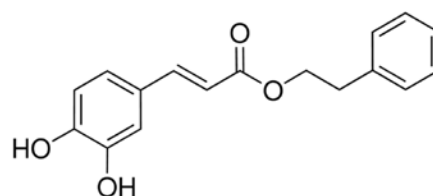


Figure 1. Chemical structure of caffeic acid phenethyl ester (CAPE).

tractant protein-1 (MCP-1) were provided by Invitrogen Life Technologies (San Diego, CA, USA). ELISA kits for rat NF- $\kappa$ B were purchased from Wkea Med Supplies Corp. (Changchun, China).

**Cell isolation, culture and identification.** HSCs were prepared by *in situ* sequential perfusion of proteinase E/collagenase II at 37°C and density-gradient centrifugation, as previously described (25,26). Briefly, the rats were anesthetized with pentobarbital and the livers were perfused first with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free solution for 10 min at 37°C, and next with 0.05% (w/v) collagenase II and 0.01% proteinase E solution for 30 min at 37°C. The digested livers were excised, suspended in D-Hanks solution and filtered through a sterile gauze. Residual hepatocytes were removed by two low-speed centrifugations (50 x g) at 4°C for 2 min. The filtered suspension was centrifuged at 540 x g at 4°C for 5 min. The HSC-enriched fraction was obtained by centrifugation with a triple-layered (9, 11 and 17%) Nycodenz cushion at 1,400 x g at 4°C for 20 min. The cells in the upper layer were washed twice and incubated, on Corstar® 6-well plastic plates, with DMEM medium containing 10 mM HEPES, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml) and 10% FBS, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cell viability was determined by Trypan blue staining. The purity of the isolated HSCs was analyzed by flow cytometry: all cells were stained with propidium iodide and the HSCs were stained with the monoclonal primary anti-desmin antibody and then with the secondary antibody FITC-anti-IgG (Abcam, Cambridge, UK). HSCs spontaneously underwent transdifferentiation to MFB-like HSCs on plastic plates, and HSCs on day 7 of the primary culture were considered as myofibroblastic cells/activated HSCs when they expressed all features of MFBs.

**Nitrite assay.** HSCs from day 7 of primary culture were trypsinized and seeded in 6-well culture plates at a density of 5x10<sup>5</sup> cells/well for 12 h. The cells were pre-treated with various concentrations of CAPE (0.0, 5.0, 10.0, 20.0 and 40.0  $\mu$ M) for 2 h, and then stimulated with LPS (100 ng/ml) for 24 h. At the end of these treatments, nitrite, the oxidation product of nitric oxide (NO), was quantified in conditioned medium from HSCs, using a spectrophotometric assay as previously described (27). Absorbance values were converted to nitrite concentrations using standard curves obtained from spectrophotometric analysis of known concentrations of potassium nitrite, prepared in the same culture medium.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR).** To analyze the expression of genes coding for pro-inflammatory mediators, HSCs were treated as described

in the nitrite assay. For the analysis of the collagen gene, HSCs from days 3 and 7 (activated HSCs) of the primary culture were used. HSCs were pre-treated with various concentrations of CAPE for 2 h, then treated with 100 ng/ml LPS, 5 ng/ml TGF- $\beta$ 1 or the vehicle [phosphate-buffered saline (PBS)] for 24 h. At the end of the treatment, total RNA from the HSCs was extracted using TRIzol reagent. The concentration and purity of total RNA was spectrophotometrically assessed at 260 and 280 nm. The RNA was reverse transcribed into cDNA using a M-MuLV reverse transcriptase with RNase inhibitor. Oligonucleotide primers for quantitative PCR were designed with the freely available Primer Premier 5.0 software, based on the mRNA sequences of MCP-1, IL-6, inducible nitric oxide synthase (iNOS), collagen type I  $\alpha$ 1 (col1A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. The primer sequences were as follows: col1A1 forward, 5'-TGGTTTCCCTGGTGCTGATG-3' and reverse, 5'-CTGCCAGTGAGACCCTTGG-3'; iNOS forward, 5'-CAGGTCGGCCATTACTGTGT-3' and reverse, 5'-CGATGCACAACTGGGTGAAC-3'; MCP-1 forward, 5'-CTGTGCTGACCCCAATAAGGA-3' and reverse, 5'-GCTTGAGGTGGTTGGGAAAAG-3'; IL-6 forward, 5'-ATGAGAAAAGAGTTGTGCAATGG-3' and reverse, 5'-GGAAGCTCCAGAAGACCAGAGC-3'; and GAPDH forward, 5'-CCCAGAACATCATCCCTGCAT-3' and reverse, 5'-CATACTTGGCAGGTTTCTCCA-3'. PCR reactions were carried out on an ABI 7500 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). We prepared on ice a reaction mixture containing 1  $\mu$ l cDNA, 12.5  $\mu$ l Platinum SYBR-Green qPCR SuperMix-UDG and 1  $\mu$ l of each primer, and added H<sub>2</sub>O to a final volume of 25  $\mu$ l. The reaction conditions included an initial step at 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec, 58°C for 10 sec and 72°C for 10 sec. Each sample was analyzed in duplicate PCR reactions. We analyzed the qRT-PCR data using the comparative Ct ( $\Delta\Delta$ CT) method, as previously described (28).

**Cytokine ELISA.** MCP-1 and IL-6 protein concentrations were measured in HSC-conditioned medium using available ELISA kits, according to the manufacturer's instructions. Optical densities (OD) were measured using an ELISA plate reader (Bio-Rad, Hercules, CA, USA) at 450 nm wavelength.

**Western blot analysis.** The preparation of whole-cell lysates was carried out as previously described (29). Cytosolic extracts were prepared using the Cytoplasmic Protein Extraction kit (BioTeke, Beijing, China) according to the instructions of the manufacturer. Western blot analysis was performed as described in a previous study (30). Equal amounts of protein were resolved on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. The blot was then blocked with 5% non-fat dry milk and probed overnight with primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. The primary rabbit antibodies used were the following: anti-p-IkBa, anti-IkBa and anti-GAPDH (all at 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-iNOS (1:200) and anti-col1A1 antibodies (1:2,000) (both from LifeSpan BioSciences Inc., Seattle, WA, USA). Protein bands were visualized using a chemiluminescent reagent.

**NF- $\kappa$ B translocation assay.** The cells were pre-treated with various concentrations of CAPE for 2 h and then treated with 100 ng/ml LPS for 30 min. At the end of the treatment, nuclear extracts were isolated by using the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). Briefly, cells were collected in a PBS/phosphatase inhibitors solution and lysed in a buffer containing DTT and a cocktail of protease inhibitors as per the manufacturer's recommendations. Solubilized proteins were then separated from cell debris by centrifugation at 14,000  $\times$  g for 30 min. The protein concentration of the nuclear fraction was measured, and the protein content was adjusted to obtain the same concentration in all the samples. The translocation of NF- $\kappa$ B was measured by the corresponding ELISA kit following the manufacturer's recommendations.

**Statistical analysis.** All data are presented as the means  $\pm$  SD. Statistical analysis was performed using SPSS software 13.0, with a P-value <0.05 considered to indicate a statistically significant difference.

## Results

**Activation and identification of HSCs.** Freshly isolated HSCs from rats were cultured on plastic plates in medium containing 10% FBS. In phase contrast microscopic observations, the HSCs spontaneously underwent transdifferentiation into MFB-like HSCs from day 2-3, and became fully activated on day 7 of primary culture. Freshly isolated HSCs were spherical. Following culture for 48 h, the cells were spindle-shaped or spherical (Fig. 2A). On day 7 of primary culture, HSCs were fully spreading, presenting stellate or polygonal forms (Fig. 2B). The yield rate of HSCs was (1-6)  $\times 10^7$ /rat liver, and cell viability was >90%, as determined by Trypan blue staining. Flow cytometric analysis revealed that the percentage of desmin-positive cells was 94.7% of the freshly isolated HSCs (Fig. 2C), indicating that an adequate level of purity was achieved for the subsequent experiments.

**Inhibitory effects of CAPE on LPS-induced nitrite and iNOS production in HSCs.** LPS stimulation resulted in a 11.5-fold increase in the nitrite level compared to the vehicle-treated control. CAPE inhibited this induction in a dose-dependent manner (Fig. 3A), with 20 or 40  $\mu$ M of CAPE significantly inhibiting LPS-induced nitrite production ( $P < 0.01$ ). In lactate dehydrogenase (LDH) assays, performed so as to assess the effect of CAPE on cell toxicity, there were no significant differences between the groups, indicating that CAPE treatment was not toxic under the tested concentrations (data not shown). As shown by western blot analysis, a very weak iNOS band was detected in the vehicle-treated control (Fig. 3B). By contrast, iNOS protein expression was markedly increased in the LPS-stimulated cells and it was inhibited in the cells pre-treated with CAPE. Densitometric analysis (Fig. 3C) revealed that iNOS expression was significantly increased upon LPS treatment compared to treatment with the vehicle, and CAPE pre-treatment markedly and significantly reduced the LPS-induced iNOS expression compared to LPS treatment alone ( $P < 0.01$ ).

**CAPE decreases the transcript levels of pro-inflammatory mediator genes in HSCs.** An earlier study suggested that

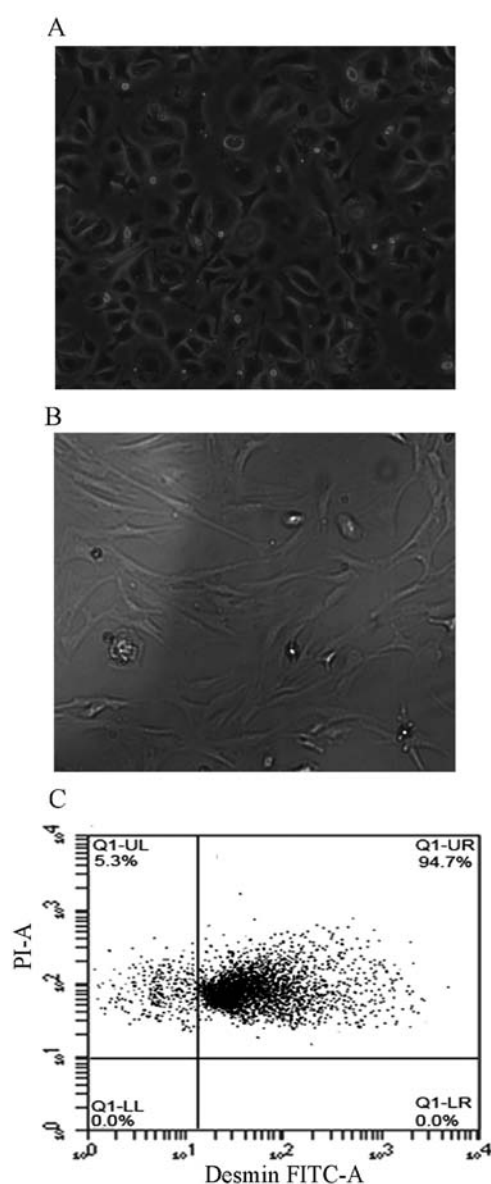


Figure 2. Activation and identification of hepatic stellate cells (HSCs). (A) Primary HSCs cultured for 48 h. The cells are spindle-shaped or spherical (x200). (B) Culture-activated HSCs. HSCs are fully spreading, presenting stellate or polygonal forms on day 7 of primary culture (x200). (C) Cell purity as assessed by flow cytometry. Freshly isolated HSCs were stained with monoclonal anti-desmin/FITC-anti-IgG antibodies. A total of 94.7% cells was estimated to be desmin-positive.

LPS/TLR4 signaling in HSCs constitutes the molecular link between inflammation and liver fibrosis (6). It was thus interesting to examine whether CAPE inhibits the LPS-induced expression of genes coding for pro-inflammatory mediators. The levels of MCP-1, IL-6 and iNOS transcripts (mRNA) were quantified in the HSCs by qRT-PCR. Upon exposure to 100 ng/ml of LPS for 24 h, the HSCs developed a strong pro-inflammatory phenotype, as shown by the increased mRNA expression of MCP-1, IL-6 and iNOS (Fig. 4). CAPE pre-treatment significantly reduced the LPS-induced mRNA expression of MCP-1, IL-6 and iNOS in a dose-dependent manner (Fig. 4). These data demonstrate that CAPE attenuates the pro-inflammatory phenotype of LPS-stimulated HSCs.

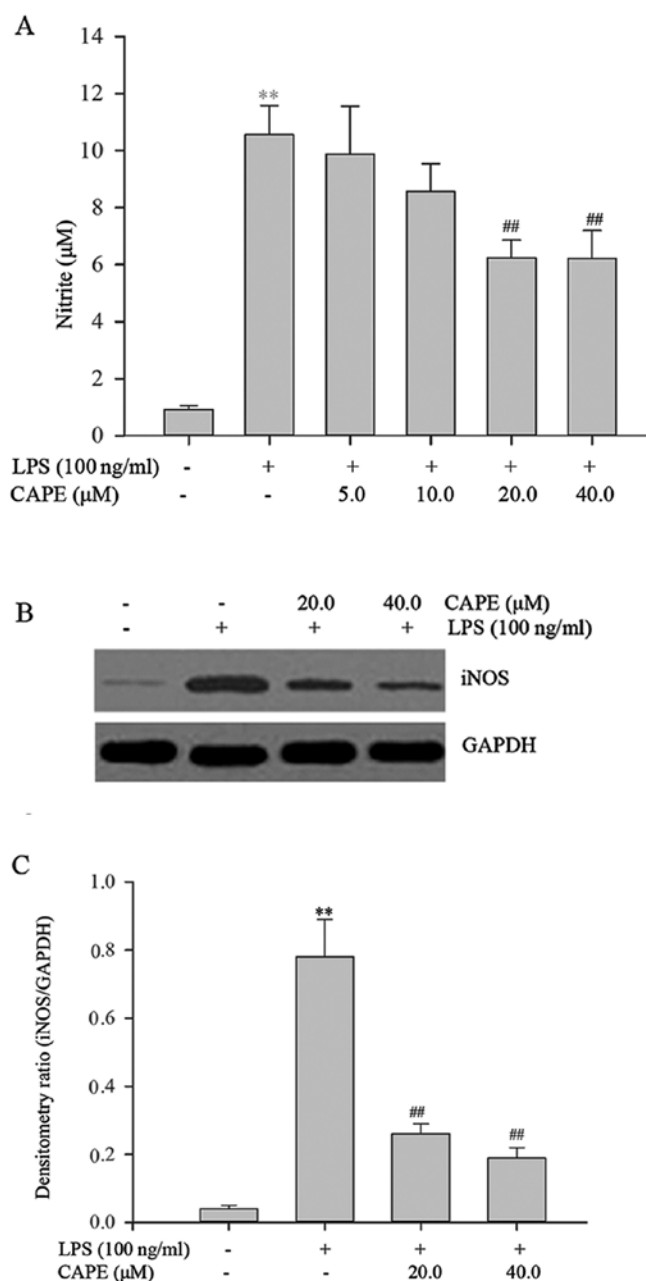


Figure 3. Effects of caffeic acid phenethyl ester (CAPE) on nitrite and inducible nitric oxide synthase (iNOS) production. Activated hepatic stellate cells (HSCs) were subcultured in 6-well plastic plates at a density of  $5 \times 10^5$ /well for 12 h, were pre-treated with various concentrations of CAPE or the vehicle for 2 h, and treated with lipopolysaccharide (LPS) (100 ng/ml) or the vehicle for 24 h. (A) CAPE decreases nitrite concentration in HSC-conditioned medium, as shown by analysis of spectrophotometric data. (B) Western blot analysis of iNOS protein, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. (C) Ratios of iNOS/GAPDH band intensities from densitometry analysis. Data are presented as the mean  $\pm$  SD values of duplicate measurements from at least 3 independent experiments. \*\* $P < 0.01$  vs. vehicle-treated control; ## $P < 0.01$  vs. LPS treatment.

*CAPE decreases pro-inflammatory cytokine release from LPS-stimulated HSCs.* In response to LPS stimulation, the HSCs secreted considerable amounts of MCP-1 and IL-6, with a 2.6- and 2.2-fold increase relative to the vehicle-treated control, respectively. Following treatment with 20 or 40  $\mu$ M CAPE, the production of IL-6 and MCP-1 proteins by LPS-stimulated

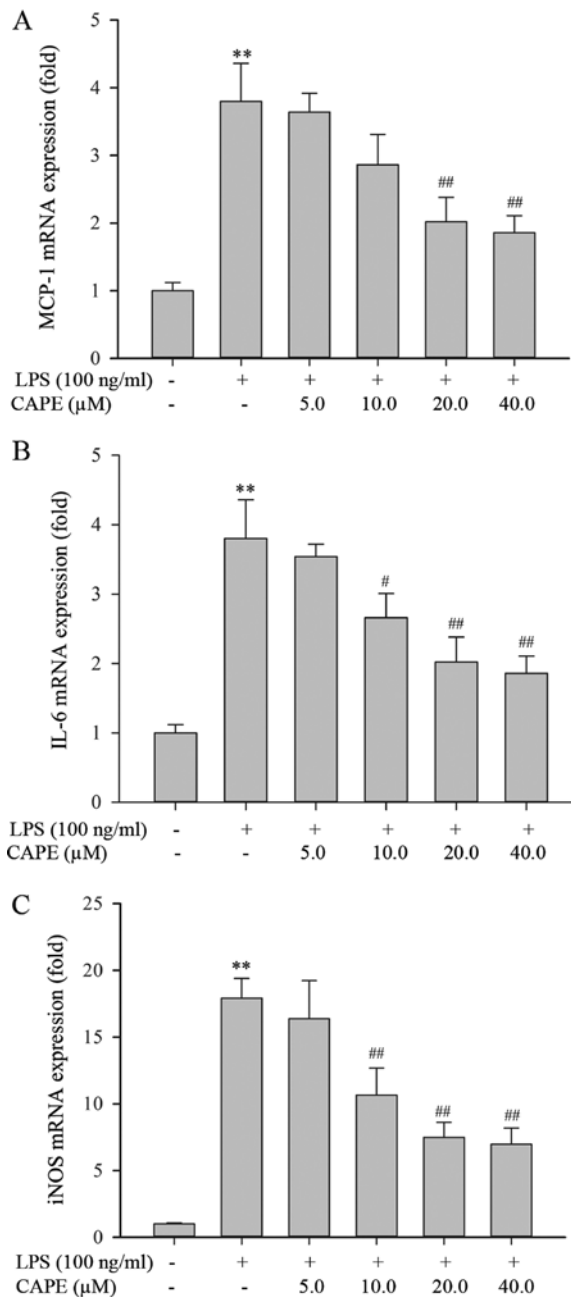


Figure 4. Caffeic acid phenethyl ester (CAPE) decreases the mRNA levels of genes coding for pro-inflammatory mediators in hepatic stellate cells (HSCs) in a dose-dependent manner. Activated HSCs were subcultured in 6-well plastic plates at a density of  $5 \times 10^5$ /well for 12 h, were pre-treated with various concentrations of CAPE or the vehicle for 2 h, and then treated with lipopolysaccharide (LPS) (100 ng/ml) or the vehicle for 24 h. CAPE dose-dependently inhibits (A) monocyte chemoattractant protein-1 (MCP-1), (B) interleukin-6 (IL-6) and (C) inducible nitric oxide synthase (iNOS) transcription, as shown by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Data are presented as the mean  $\pm$  SD values of duplicate measurements from at least 3 independent experiments. \*\* $P < 0.01$  vs. vehicle-treated control; ## $P < 0.01$  vs. LPS treatment.

HSCs was significantly decreased (Fig. 5). This result is consistent with the data obtained for mRNA expression by qRT-PCR (Fig. 5).

*CAPE attenuates TGF- $\beta$ 1-induced collagen synthesis in LPS-stimulated HSCs.* Kupffer cell-derived TGF- $\beta$ 1 is a highly potent profibrogenic growth factor that activates collagen

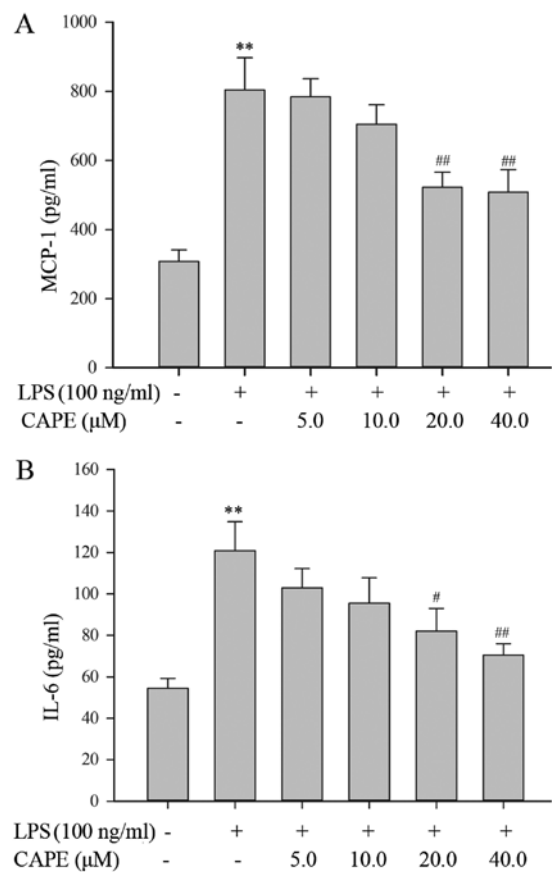


Figure 5. Caffeic acid phenethyl ester (CAPE) decreases lipopolysaccharide (LPS)-induced monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) release. Activated hepatic stellate cells (HSCs) were subcultured in 6-well plastic plates at a density of  $5 \times 10^5$ /well for 12 h, were pre-treated with various concentrations of CAPE or the vehicle for 2 h, and treated with LPS (100 ng/ml) or vehicle solution for 24 h. MCP-1 and IL-6 were then detected in conditioned medium using an enzyme-linked immunosorbent assay (ELISA). CAPE inhibits (A) MCP-1 and (B) IL-6 release from LPS-stimulated HSCs. \*\* $P < 0.01$  vs. vehicle-treated control; # $P < 0.05$  and ## $P < 0.01$  vs. LPS treatment.

synthesis in HSCs *in vivo* (6,31). In the present study, we investigated the effects of CAPE on the TGF- $\beta$ 1-induced fibrogenic phenotype of LPS-stimulated HSCs showing a strong pro-inflammatory phenotype. LPS alone did not directly induce a fibrogenic phenotype, since collagen type 1 mRNA and protein levels were substantially unaffected by LPS stimulation (Fig. 6). However, LPS stimulation significantly enhanced the TGF- $\beta$ 1-induced mRNA expression of  $\alpha 1(I)$  in the HSCs from days 3 and 7 of primary culture, and significantly amplified the TGF- $\beta$ 1-induced collagen synthesis in the HSCs from day 3 of primary culture, as shown by qRT-PCR and western blot analysis, respectively (Fig. 6). Upon CAPE pre-treatment, the LPS-induced upregulation of the transcription (mRNA expression) and translation (protein synthesis) of  $\alpha 1(I)$  was reversed in the HSCs from day 3 and 7 of primary culture (Fig. 6). These data suggest that CAPE attenuates the fibrogenic phenotype of LPS-stimulated HSCs by reducing their sensitization to TGF- $\beta$ 1.

*CAPE inhibits the LPS-induced phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B translocation in HSCs.* NF- $\kappa$ B is an important transcription factor, orchestrating the production of pro-inflammatory

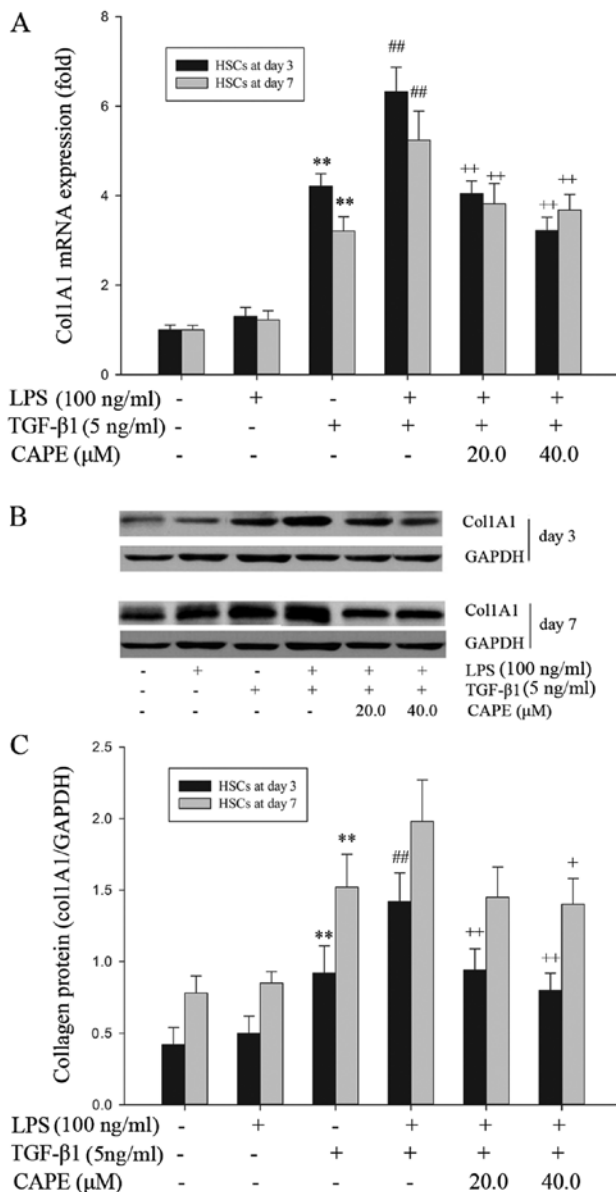


Figure 6. Caffeic acid phenethyl ester (CAPE) attenuates transforming growth factor-β1 (TGF-β1)-mediated collagen synthesis in lipopolysaccharide (LPS)-stimulated hepatic stellate cells (HSCs). HSCs from days 3 and 7 of primary culture were subcultured in 6-well plastic plates at a density of  $5 \times 10^5$ /well for 12 h, were pre-treated with various concentrations of CAPE or the vehicle for 2 h, and treated with LPS (100 ng/ml), TGF-β1 (5 ng/ml) or the vehicle for 24 h. (A) CAPE decreased the mRNA level of TGF-β1-induced collagen type I α1 (col1A1) in LPS-treated HSCs, as shown by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (B) Western blot analysis of col1A1 protein expression. (C) CAPE significantly reduced TGF-β1-induced col1A1 protein expression in LPS-treated HSCs. Data are presented as col1A1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) band intensity ratios from densitometry analysis. \*\* $P < 0.01$  vs. vehicle-treated control; ## $P < 0.01$  vs. LPS treatment only; ++ $P < 0.01$  vs. LPS and TGF-β1 treatment.

mediators, as well as regulating a variety of important cellular functions. To correlate the attenuation of pro-inflammatory and fibrogenic phenotypes by CAPE in HSCs with downstream elements of the LPS/TLR4 signal transduction pathway, we examined the phosphorylation and degradation of IκBα by western blot analysis and the NF-κB nuclear translocation by an ELISA assay. In response to LPS, the phosphorylation of IκBα was markedly enhanced and IκBα was markedly

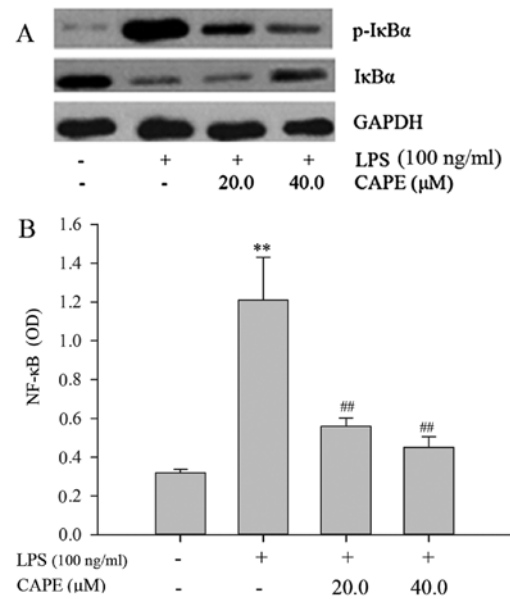


Figure 7. Caffeic acid phenethyl ester (CAPE) inhibits lipopolysaccharide (LPS)-induced phosphorylation of IκBα and nuclear factor-κB (NF-κB) activity in hepatic stellate cells (HSCs). Activated HSCs were subcultured in 6-well plastic plates at a density of  $5 \times 10^5$ /well for 12 h, were pre-treated with various concentrations of CAPE or the vehicle for 2 h, and treated with 100 ng/ml LPS or the vehicle for 30 min. (A) CAPE inhibited LPS-induced phosphorylation of IκBα. Cytosolic IκBα protein was detected by western blot analysis using anti-p-IκBα, anti-IκBα and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (GAPDH serving as the loading control). (B) CAPE inhibited LPS-induced NF-κB nuclear translocation in HSCs. Nuclear extracts were prepared and the NF-κB (p65) protein presence in the nuclear extract was detected by enzyme-linked immunosorbent assay (ELISA). Data are presented as the mean  $\pm$  SD values of at least 3 independent experiments. \*\* $P < 0.01$  vs. vehicle-treated control; ## $P < 0.01$  vs. LPS treatment.

degraded. CAPE pre-treatment inhibited the phosphorylation of IκBα and consequently prevented its degradation (Fig. 7A). LPS induced a 3.8-fold increase in the nuclear translocation of NF-κB. Pre-treatment of the cells with 20 and 40 μM of CAPE significantly inhibited the LPS-induced NF-κB translocation to the nucleus by 53.7 and 62.8%, respectively ( $P < 0.01$ , compared to LPS treatment) (Fig. 7B). This result demonstrates that the mechanism underlying the attenuation of the pro-inflammatory and fibrogenic phenotypes of HSCs by CAPE involves the inhibition of NF-κB signaling.

## Discussion

Hepatic fibrosis is tightly linked to chronic liver inflammation in all individuals with liver disease and in experimental models of fibrogenesis. The molecular link between inflammation and liver fibrosis was shown to be TLR4 signaling, which promotes myofibroblast activation and modulates TGF-β signaling in HSCs (6,32). Liver fibrogenesis is associated with increased intestinal permeability (33). In hepatic sinusoids, intestine-derived bacterial products absorbed through the gastrointestinal wall may reach parenchymal and non-parenchymal hepatic cells, and they are recognized by pattern recognition receptors (PRRs) that bind to conserved microbial structures. Typically, immune cells, such as monocytes and

Kupffer cells in the liver express basal levels of PRRs to allow an immediate and unspecific inflammatory response to bacterial products (34,35). However, accumulating evidence indicates that quiescent or activated HSCs also express TLR4, one of the well-characterized PRRs, and can thereby respond to low concentrations of LPS (6,36,37).

Our results demonstrated that LPS induced an intense pro-inflammatory response in HSCs, leading to a marked increase in the nitrite level and to an upregulation in the levels of pro-inflammatory mediators. This result is consistent with previous studies showing that bacterial products induce a strong pro-inflammatory phenotype of HSCs, triggering the release of pro-inflammatory mediators and thus, contributing to tissue injury and liver fibrosis (38). The HSC-derived factors lead to chemoattraction of bone marrow-derived monocytes and the accumulation of Kupffer cells in the liver (6,39), or act in an autocrine manner to activate HSCs (39,40).

To the best of our knowledge, the present study is the first to demonstrate that CAPE attenuates the pro-inflammatory and fibrogenic phenotypes of LPS-stimulated HSCs. CAPE significantly reduced the production of the NO free radical, nitrite, and that of the pro-inflammatory mediators, MCP-1, IL-6 and iNOS, in LPS-stimulated HSCs *in vitro*. Previous studies have demonstrated that CAPE exhibits a plethora of important biological properties, such as potent anti-inflammatory, anti-tumor and antioxidant activities, and attenuates inflammation and lipid peroxidation (23,41,42). Thus, it may be possible that CAPE can decrease liver inflammation and fibrosis *in vivo* by downregulating the secretion of chemokines from HSCs, thereby reducing the chemoattraction of monocytes and Kupffer cells in the liver.

As regards collagen synthesis, LPS did not directly induce *col1A1* mRNA transcription and protein expression in HSCs *in vitro*. *In vivo*, the development of hepatic fibrosis involves a cross-talk between HSCs and other cell types, particularly Kupffer cells. The Kupffer cell-derived TGF- $\beta$  protein plays a crucial role in promoting HSC activation and fibrogenesis (31,43). The results from the present study demonstrated that LPS treatment led to an increase in TGF- $\beta$ 1-induced collagen production, and CAPE decreased the sensitivity of HSCs to TGF- $\beta$ 1. This effect may be mediated by the downregulation of the TGF- $\beta$  pseudoreceptor, the bone morphogenetic protein and activin membrane bound inhibitor (BAMBI), leading to HSC activation and sensitivity to TGF- $\beta$  (6,7). Our data demonstrated that CAPE decreased the sensitivity of HSCs to TGF- $\beta$ 1, indicating that CAPE may also interfere with TGF- $\beta$  receptor signaling in LPS-stimulated HSCs.

The crucial role of LPS/TLR4 signaling in HSCs during liver fibrogenesis has been demonstrated in previous studies (6,13). NF- $\kappa$ B is a downstream element of the LPS/TLR4 signal transduction cascade, and one of the most ubiquitous transcription factors, regulating the expression of genes involved in cellular proliferation, inflammatory responses and cell adhesion (44,45). In the cytosol, NF- $\kappa$ B remains inactive by forming a complex with the inhibitory protein I $\kappa$ B $\alpha$ . In response to stimuli, I $\kappa$ B $\alpha$  kinases (I $\kappa$ Ks) mediate I $\kappa$ B $\alpha$  phosphorylation, the dissociation of the NF- $\kappa$ B/I $\kappa$ B $\alpha$  complex, and the activation of NF- $\kappa$ B, which then translocates to the nucleus to activate specific target genes (46,47). Activation of NF- $\kappa$ B in hepatic cells was

shown to correlate with hepatic inflammation and fibrosis (48). Our data show that CAPE treatment inhibits LPS-induced phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B nuclear translocation in HSCs. These results indicate that the anti-inflammatory and anti-fibrogenic effects of CAPE may associate with the inhibition of NF- $\kappa$ B translocation, and result in the downregulation of pro-inflammatory genes in LPS-stimulated HSCs.

In conclusion, the present study demonstrates that CAPE attenuates the LPS-induced pro-inflammatory and fibrogenic phenotypes in rat HSCs via its effects on NF- $\kappa$ B signaling. This finding provides new insight into the treatment of hepatic fibrosis through the regulation of the TLR4 signaling pathway.

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