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ORIGINAL ARTICLE

Basic Study

Caffeic acid phenethyl ester up-regulates antioxidant levels in hepatic stellate cell line T6 *via* an Nrf2-mediated mitogen activated protein kinases pathway

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Abstract

AIM

To investigate the antioxidant effect of caffeic acid phenethyl ester (CAPE) in hepatic stellate cell-T6 (HSC-T6) cells cultured *in vitro* and the potential mechanisms.

METHODS

HSC-T6 cells were cultured *in vitro* and treated with various concentrations of CAPE for 24, 48 and 72 h, respectively. Cell proliferation was investigated using the MTT assay, and cell ultrastructural alterations were observed by transmission electron microscopy. Flow cytometry was employed to investigate the effects of CAPE on apoptosis and the levels of reactive oxygen species in HSC-T6 cells cultured *in vitro*. An enzyme immunoassay instrument was used to evaluate antioxidant enzyme expression. The effect on α-smooth muscle actin was shown using immunofluorescence. Gene and protein levels of Nrf2, related factors, and mitogen activated protein kinases (MAPKs), in HSC-T6 cells were detected using RT-PCR and Western blot, respectively.

RESULTS

CAPE inhibited the proliferation and activation of HSC-T6 cells cultured *in vitro*. CAPE increased the antioxidant levels and the translocation of Nrf2 from

the cytoplasm to the nucleus in HSC-T6 cells. Moreover, the phosphorylation of MAPKs in cells decreased in response to CAPE. Interestingly, CAPE-induced oxidative stress in the cells was significantly attenuated by pretreatment with MAPKs inhibitors.

CONCLUSION

CAPE inhibits cell proliferation and up-regulates the antioxidant levels in HSC-T6 cells partly through the Nrf2-MAPKs signaling pathway.

Key words: Caffeic acid phenethyl ester; Liver fibrosis; Antioxidation; Nrf2; Mitogen activated protein kinases

Core tip: Liver fibrosis is a pathological response to hepatocyte injury, including oxidative stress, which is a primary mechanism of liver damage. Caffeic acid phenethyl ester (CAPE) is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. It has been used in the treatment of several diseases. In this study, we investigated the antioxidant effect of CAPE in HSC-T6 cells and its potential mechanism. Our results demonstrated that CAPE inhibited cell proliferation and up-regulated the antioxidant levels in HSC-T6 cells partly through the Nrf2-MAPKs signaling pathway.

Yang N, Shi JJ, Wu FP, Li M, Zhang X, Li YP, Zhai S, Jia XL, Dang SS. Caffeic acid phenethyl ester up-regulates antioxidant levels in hepatic stellate cell line T6 *via* an Nrf2-mediated mitogen activated protein kinases pathway. *World J Gastroenterol* 2017; 23(7): 1203-1214 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i7/1203.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i7.1203

INTRODUCTION

Liver fibrosis is a pathological response to hepatocyte injury, including injury caused by viral infection, chronic inflammation, and other factors such as oxidative stress, which is a primary mechanism of liver damage[1,2]. Oxidative stress can stimulate the activation of hepatic stellate cells (HSCs) through paracrine and autocrine mechanisms, leading to the formation of liver fibrosis[1,2]. HSC-T6 cells, a well-differentiated transformed cell line[3,4], were used in this study to observe the role of HSC in liver fibrosis.

Caffeic acid phenethyl ester (CAPE) is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. It has been used in the treatment of several diseases[5-7]. Our previous studies have shown that CAPE inhibited liver fibrosis in rats due to its ability to suppress oxidative stress[8].

Antioxidants play a pivotal role in the development of liver fibrosis[1]. Several antioxidative stress factors have been identified, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and nonenzymatic compounds [vitamin E, carotene, and glutathione-S-transferase (GSTs)], in liver tissue[9,10]. SOD, CAT, and GSTs are recognized enzymes that are closely related to nuclear factor-erythroid 2 related factor 2 (Nrf2)[11,12]. Nrf2 is an important transcription factor regulating the expression of antioxidative stress factors. Nrf2 binds to an antioxidant response element (ARE) in the promoter region of genes encoding several phase-II detoxifying/antioxidant enzymes and related stress-responsive proteins, including SOD, CAT and GSTs[12]. Several studies have confirmed that Nrf2 is involved in the signaling pathway of mitogen activated protein kinase (MAPKs) in nuclear translocation[13]. MAPKs, including the ERK1/2, JNK1/2, and p38 signaling pathways, play a critical role in regulating the oxidative stress response in various types of cells. However, it is still largely unknown whether CAPE can reduce the expression of these factors *via* the Nrf2-mediated MAPKs signaling pathway in HSCs.

In this study, we show that CAPE inhibits the proliferation and activation, but increases apoptosis, of HSC-T6 cells *in vitro*. CAPE up-regulates the antioxidant capacity in HSC-T6 cells through the Nrf2-mediated MAPKs signaling pathway.

MATERIALS AND METHODS

Reagents

The cell apoptosis kit and SB203580 were purchased from Joincare Pharmaceutical Industry Group Co., Ltd. (Nanjing, China). SP600125 and PD98059 were obtained from Sigma-Aldrich (St. Louis, United States) and Cell Signaling Technology (Boston, United States), respectively. CAPE (Sigma-Aldrich, St. Louis, MO, United States) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, United States) to 40 mmol/L as a stock solution and stored at -20 ℃. Control flasks or plates contained DMSO at an equivalent dilution in cultures containing CAPE.

Cell culture

HSC-T6 cells were purchased from Joincare Pharmaceutical Industry Group Co., Ltd. (NanJing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Utah, United States) supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone, Utah, United States), 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, United States), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, United States), 2 mmol/mL L-glutamine (Sigma-Aldrich, St. Louis, United States), and 100 U/mL DNase I (Sigma-Aldrich, St. Louis, United States) at 37 ℃ in a 5% (v/v) CO_2 humidity atmosphere. Cells adhering to the culture flask in logarithmic phase were trypsinized and subjected to

Table 1 Primers used for real-time PCR analysis

Gene	Primer sequence	Tm (℃)	Size (bp)	Source
Nrf2				
F	5′-CTGCCATTAGTCAGTCGCTCTC-3′	57.3	22	Rat
R	5′-TCGGCTGGGACTTGTGTTC-3′	57.7	19	Rat
SOD1				
F	5′-GCTTCTGTCGTCTCCTTGCT-3′	55.7	20	Rat
R	5′-CTCGAAGTGAATGA CGCCCT-3′	55.8	20	Rat
CAT				
F	5′-TGGCTATGGCTCACACACCTTC-3′	59.3	22	Rat
R	5′-GAGGCCA TAATCCGGGTCTTC-3′	57.7	21	Rat
GSTs				
F	5′-GTGGAGATTGACGGGATGAA -3′	54.2	20	Rat
R	5′-CGGTCTTGGCTTCTCTTTGG -3′	56.0	20	Rat
β-actin				
F	5′-GGAGATTACTGCCCTGGCTCCTA-3′	60.2	23	Rat
R	5′-GACTCATCGTACTCCTGCTTGCTG-3′	59.3	24	Rat

passage as usual for further experimentation.

Cell viability assay

The tetrazolium dye colorimetric test [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide, MTT, Sigma-Aldrich, St. Louis, MO, United States] was used to determine the viability of HSC-T6 cells. The MTT assay is based on the ability of functional mitochondria to catalyze the reduction of MTT to insoluble purple formazan, the concentration of which can be measured spectrophotometrically, as described previously[14]. HSC-T6 cells were incubated on a 96-well plate at a density of 5×10^5 cells/well for 24 h and treated with different concentrations of CAPE (0, 5, 10, 15, 20, 40, 60, 80 and 100 μmol/L). MTT solution (0.5 mg/mL) was then added to each well. The MTT solution was replaced with DMSO to dissolve the blue formazan crystals. Four hours later, absorbance was measured at 490 nm using a microplate reader (BioTekInstruments, United States) and the percentage viability was calculated.

Transmission electron microscopy

Cells in the logarithmic phase were treated with a range of CAPE (5, 10 and 15 μmol/L) for 24 h or established as controls. At the end of the treatment, cells were washed twice with phosphate buffered solution (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HPO_4, 1.4 mmol/L KH_2PO_4, pH 7.4), fixed in 2.5% (v/v) glutaraldehyde followed by 1% (v/v) perosmic acid, and dehydrated in an ethanol series. Ultrathin sections were placed on 400-mesh grids and double-stained with uranyl acetate and lead citrate. The ultrastructures were observed using a transmission electron microscope (HITACHI-H7650, Tokyo, Japan).

Apoptosis assay

Cell apoptosis was determined using Annexin V-FITC and PI double staining (KaiJi, Nanjing, China), as described previously[15]. Cells grown in logarithmic phase were treated with a range of CAPE (5, 10 and 15 μmol/L) for 24 h or established as controls. At the end of the treatment, cells were collected by centrifugation and washed twice with PBS. Cell pellets were resuspended in 0.5 mL PBS and fixed in 5 mL 70% (v/v) ice-cold ethanol at 4 ℃ for 24 h. After resuspension in 1 mL PBS, the cells were incubated with ribonuclease A (Rnase A, 20 mg/L, Sigma-Aldrich) and propidium iodide (PI, 50 mg/L, Sigma-Aldrich) for 1 h at 37 ℃ in the dark. The stained cells were analyzed using a FACscan flow cytometer in combination with BD lysis II software (CALIBUR, BD, United States).

Detection of SOD and CAT activity and GSH content

SOD and CAT activity and GSH content were measured using a SOD protein reagent kit (Bio-Rad, Hercules, CA, United States), CAT protein reagent kit (Bio-Rad, Hercules, CA, United States), and GSH protein reagent kit (Bio-Rad, Hercules, CA, United States), respectively. Briefly, HSC-T6 cells grown in logarithmic phase were treated with a range of CAPE (5, 10 and 15 μmol/L) for 24 h. The cells were washed twice with PBS, collected by centrifugation, and lysed in cell disrupter buffer (Bio-Rad, Hercules, CA, United States). After centrifugation, the protein concentrations of the supernatant were determined using a protein reagent kit. The optical density (OD) of SOD, GSH, and CAT was measured using a spectrophotometer (ND-1000, Thermo Fisher, United States) at wavelengths of 450 nm, 405 nm and 420 nm to generate the standard curve and calculate the concentrations of SOD, GSH and CAT, respectively.

Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, United States) and reverse transcription was carried out using an RT-PCR kit (Takara, Japan), as described previously[16]. Real-time PCR was performed using the SYBRE Script TM RT-PCR Kit (Takara, Japan) on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) according to the manufacturer's protocols. The primers are shown in Table 1. The comparative CT method was used to quantify the target gene expression, with β-actin used as an internal control[17].

Immunocytochemistry

The expression of α-SMA and Nrf2 in HSC-T6 cells was investigated using immunofluoresence staining[18]. Briefly, the cells were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.1% (v/v) Triton X-100 (Sigma-Aldrich). After blocking with 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) and 1% (v/v) normal donkey serum (Sigma-Aldrich), the cells were incubated with rabbit anti-α-SMA (1:500) or Nrf2 (1:500) primary antibody (Table 2) for 16 h at 4 ℃. The cells were washed and further incubated with peroxidase-conjugated anti-rabbit IgG (1:1000, Pierce Biotechnology, Rockford, United States) for 1 h. The cell nuclei were counter-

Table 2 Antibodies used for Western blot analysis

Antibody (dilution)	Clone	Species	Specificity	Source
Nrf2 (16396-1-AP 1:500)	M	Rabbit	Immunogen: ag9489	ProteinTech, United States
P38MAPK (ab32142, 1:500)	M	Rabbit	Synthetic peptide corresponding to residues near the C-terminus of human p38 MAP kinase was used as immunogen. Predicted to react with CSBP1 splice form based on sequence homology	EPITOMICS, United States
P-p38MAPK (#4511, 1:500)	M	Rabbit	Phospho-P38 MAPKinase (Thr180/Tyr182) (3D7) rabbit mAb detects endogenous levels of p38MAP kinase only when dually phosphorylated at Thr180 and Tyr182. This antibody does not cross-react with the phosphorylated forms of either p42/44 MAPK or SAPK/JNK	Cell Signaling, United States
Erk1/2 (#4695, 1:500)	M	Rabbit	P44/42 MAP kinase (137F5) rabbit mAb detects endogenous level of total P44/42 MAP kinase (Erk1/Erk2) protein. The antibody does not cross-react with JNK/SAPK or p38 MAP kinase	Cell Signaling, United States
Erk1phospho(pY204)/ Erk2phospho (pY187) (ab76299 1:500)	M	Rabbit	A phospho-specific peptide corresponding to residues surrounding tyrosine 204 of human Erk1. This antibody detects Erk1 phosphorylated at tyrosine 204	EPITOMICS, United States
SAPK/JNK (#9258 1:500)	M	Rabbit	SAPK/JNK(56G8) rabbit mAb detects endogenous levels of total SAPK/JNK protein	Cell Signaling, United States
JNK1phospho(pT183) /JNK2phospho(pT221) (ab124956 1:500)	M	Rabbit	A phospho-specific peptide corresponding to residues surrounding threonine 221 of human JNK3 was used as immunogen. The antibody detects JNK1 (pT183), JNK2 (pT183) and JNK3 (pT221)	EPITOMICS, United States
α-SMA (ab124964,1: 1000)	M	Rabbit	A synthetic peptide corresponding to N-terminus of human actin was used as immunogen	EPITOMICS, United States
Collegen-1 (bs-0578R 1:500)	P	Rabbit	KLH conjugated synthetic peptide derived from human Collagen I C-terminal propeptide	Bioss, China
β-actin (sc-47778, 1:1000)	M	Mouse	β-actin is recommended for detection of β-actin of mouse, rat, human, chicken, dog, pig, rabbit	SANTA CRUZ, United States

M: Monoclone; P: Polyclone.

stained using 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). After neutral gum mounting, fluorescence microscopy images were obtained using a confocal microscope (Ti-E, Nikon, Japan) with a laser at 405 nm and 535 nm excitation.

Western blot analysis

The expression of Nrf2, related factors, and MAPKs in HSC-T6 cells was investigated using Western blot, as described previously[16]. Briefly, the cells were harvested and re-suspended in a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA, United States) or RIPA lysis buffer [20 mmol/L Tris, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 1% (w/v) digestive phosphatase inhibitors, 1% (w/v) protease inhibitors, 1% (w/v) phenylmethyl sulfonylfluoride (PMSF), pH 7.5] (Sigma-Aldrich). The protein content was determined using a commercial protein reagent kit (Bio-Rad, Hercules, CA, United States). Equal amounts of proteins in each sample were resolved by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE, Sigma-Aldrich) electrophoresis and the proteins were transferred onto PVDF membranes (Sigma-Aldrich). After blocking with skim milk, the membranes were incubated with the specific antibodies (Table 2) for 24 h at 4 ℃. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology, Rockford, United States) for 2 h at 37 ℃. Proteins were detected with an all-enhanced chemiluminescence detection system (Syngene, United Kingdom) and quantified using a Gel-Pro Analyzer v4.0 (Media Cybernetics, L.P., United States). β-actin was used as the loading control.

Statistical analysis

The results are shown as the mean ± SD. Differences between groups were assessed by Student's *t*-test and one- or two-way ANOVA with post-hoc Duncan multiple comparisons using SPSS 13.0. A P-value < 0.05 was considered statistically significant.

RESULTS

Effect of different concentrations of CAPE on biological characteristics of HSC-T6 cells

The MTT assay results indicated that 5, 10 and 15 μmol/L CAPE treatment for 24 h did not decrease cell viability compared to the control group (P > 0.05, Figure 1A). However, 20-100 μmol/L of CAPE was cytotoxic in HSC-T6 cells (P < 0.05, Figure 1A). Similar results were obtained after 48 and 72 h (data not shown). Therefore, 5, 10 and 15 μmol/L CAPE were used for all subsequent experiments. After treatment for 24 h, the proportion of cell apoptosis increased in a concentration-dependent manner compared to the control group (Figure 1B). Transmission electron microscopy was then used to investigate the ultrastructure of apoptotic cells. In the control group,

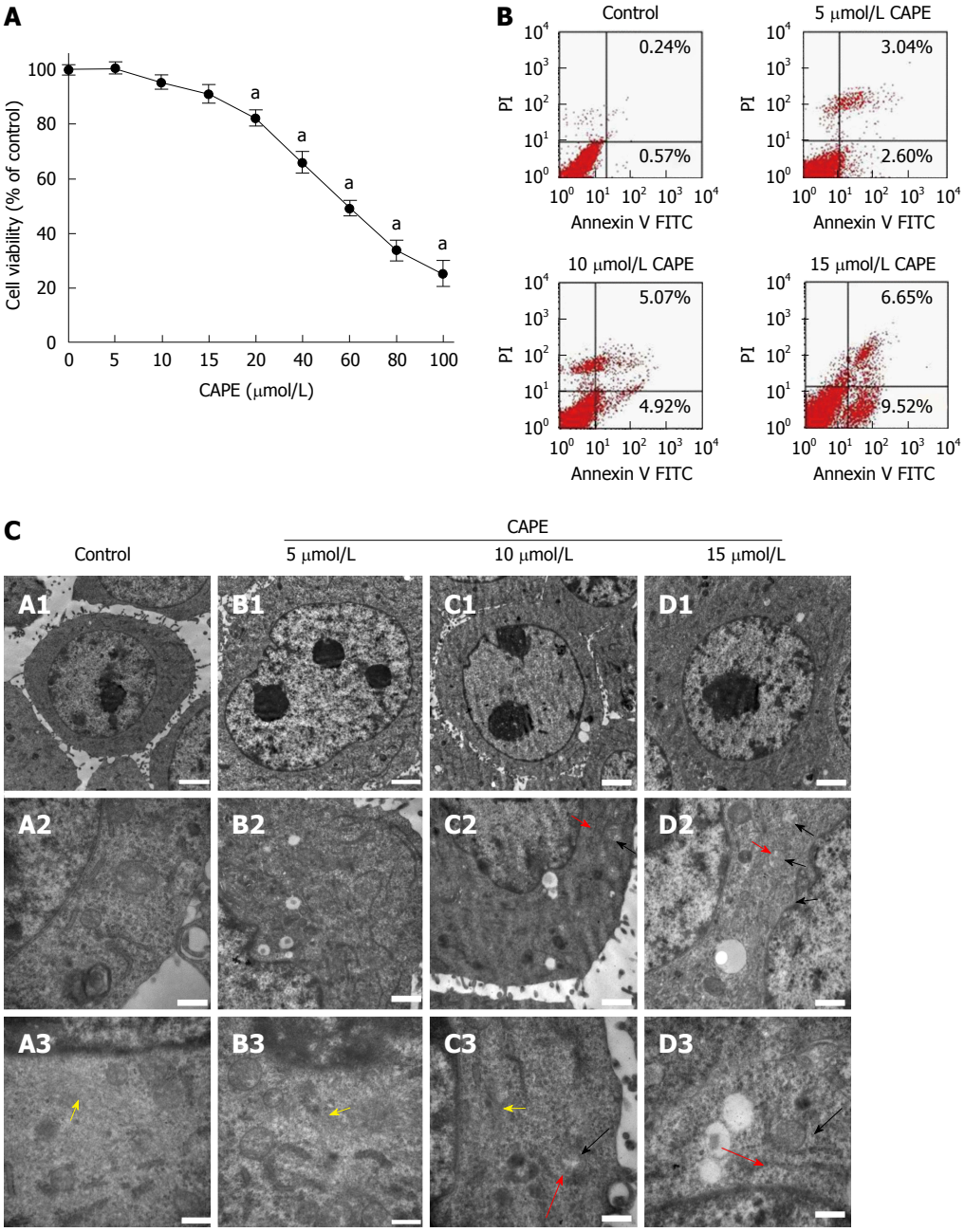

Figure 1 Effect of different concentrations of caffeic acid phenethyl ester on biological characteristics of hepatic stellate cell-T6 cells. After HSC-T6 cells were treated with CAPE(0, 5, 10, 15, 20, 40, 60, 80 and 100 μmol/L) for 24 h (A) the effect of CAPE on the viability of HSC-6 cells was detected by the MTT assay; B: Cell apoptosis was investigated using annexin V-FITC and PI and the proportion of cell apoptosis increased in a concentration-dependent manner; C: Ultrastructure of the HSC-T6 cells. The normal structure is shown in the control groups (group A). The treatment groups (groups B, C, and D) displayed prominent myofilament disarray and rupture, cytoplasmic vacuolization, and significant mitochondrial swelling (black bar: mitochondria; red bar: Endoplasmic reticulum; yellow bar: myofilament). The upper scale bar = 2 μm, the middle scale bar = 1 μm, and the lower scale bar = 0.5 μm. The data represent averages of the results of four independent experiments. [a]*P* < 0.05 *vs* control. CAPE: Caffeic acid phenethyl ester.

the cells were round with tiny villous projections observed on the cell membrane. Many plasmosomes were distributed in the nucleus; the structure of mitochondria was clear; the rough endoplasmic reticulum was streaky; and lipid droplets were found in the cytoplasm (Figure 1C-A$_{1-3}$). In the CAPE treatment groups, the growth of HSC-T6 cells was obviously inhibited; cell volume gradually declined; surface villous structure decreased or disappeared; there were fewer multiple nucleoli; there was mitochondrial swelling; the endoplasmic reticulum was slender; and a scattered distribution of lipid droplets was observed in the cytoplasm (Figure 1C-B/C/D).

α-SMA and collegen-1 protein expression in HSC-T6 cells treated with CAPE

In the control group, HSC-T6 cells were spindle-shaped and fully stained with α-SMA (Figure 2A). After treatment with 5, 10 and 15 μmol/L of CAPE for 24 h, the cell volume was lower and the cell morphology

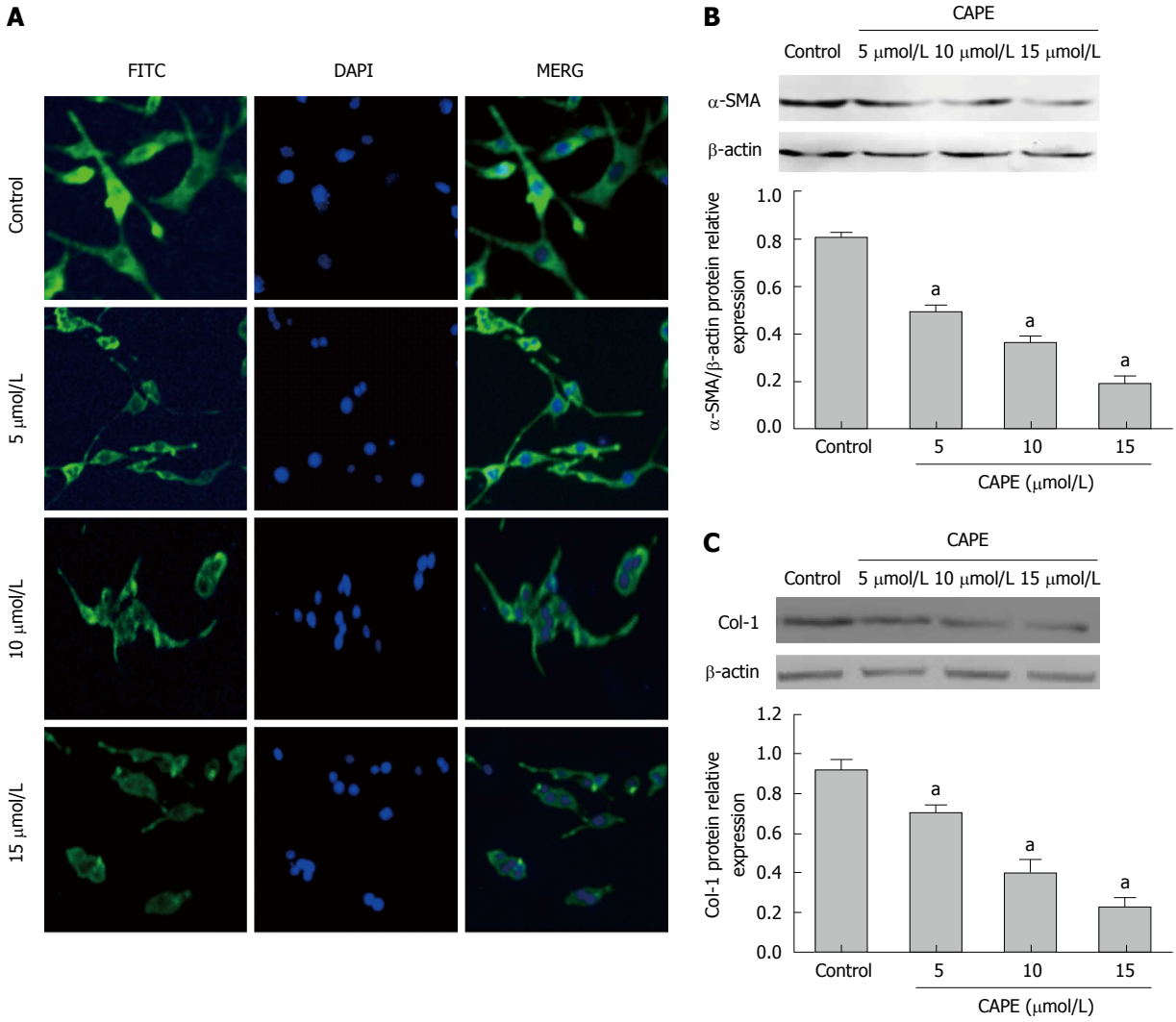

Figure 2 α-SMA and collegen-1 protein expression in hepatic stellate cell-T6 cells. After HSC-T6 cells were treated with 5 μmol/L, 10 μmol/L and 15 μmol/L CAPE for 24 h, indirect immunofluorescence (× 200) analysis of α-SMA protein expression (A) were undertaken. Western blot analysis of α-SMA and collegen-1 protein expression was also performed. Gray levels were normalized against those of the corresponding β-actin and the results are expressed relative to control (B and C). The data are the mean ± SD of three independent experiments. $^{a}P < 0.05$ *vs* control. CAPE: Caffeic acid phenethyl ester.

became round with reduced α-SMA fluorescent staining (Figure 2A). Western blot analysis showed that α-SMA and collegen-1 protein expression decreased in a dose-dependent manner in HSC-T6 cells compared to the control group ($P < 0.05$, Figure 2B and C).

Antioxidant-related indicator protein and mRNA expression in HSC-T6 cells

After treatment with CAPE for 24 h, gene and protein expression of SOD, CAT, GSH and GSTs was significantly increased in HSC-T6 cells treated with 10 μmol/L or 15 μmol/L CAPE compared to the control group ($P < 0.05$, Figure 3A and B). However, 5 μmol/L of CAPE did not affect SOD, CAT, GSH, or GSTs ($P > 0.05$, Figure 3A and B).

Effect of CAPE on Nrf2 expression in HSC-T6 cells

We observed that 10 μmol/L and 15 μmol/L of CAPE significantly increased Nrf2 gene expression in HSC-T6 cells ($P < 0.05$, Figure 4A). However, there was no alteration in Nrf2 gene expression in HSC-T6 cells in response to 5 μmol/L CAPE ($P > 0.05$, Figure 4A). Interestingly, Nrf2 protein expression in the cytosol was decreased in a dose-dependent manner ($P < 0.05$), whereas in the nucleus it was increased in a dose-dependent manner ($P < 0.05$) in HSC-T6 cells after treatment with CAPE (5, 10 and 15 μmol/L) for 24 h (Figure 4B). The nuclear/cytosol ratio of Nrf2 protein levels was significantly higher in CAPE-treated HSC-T6 cells than in the control group ($P < 0.05$, Figure 4B). Indirect immunofluorescence showed that Nrf2 protein translocated from the cytosol to the nucleus in HSC-T6 cells in the 15 μmol/L CAPE treatment group compared to the control group (Figure 4C). These results suggest that CAPE induces the activation and nuclear transcription of Nrf2.

CAPE up-regulates antioxidant levels through an Nrf2-mediated MAPKs signaling pathway in HSC-T6 cells

The phosphorylation levels of ERK1/2, p38MARK

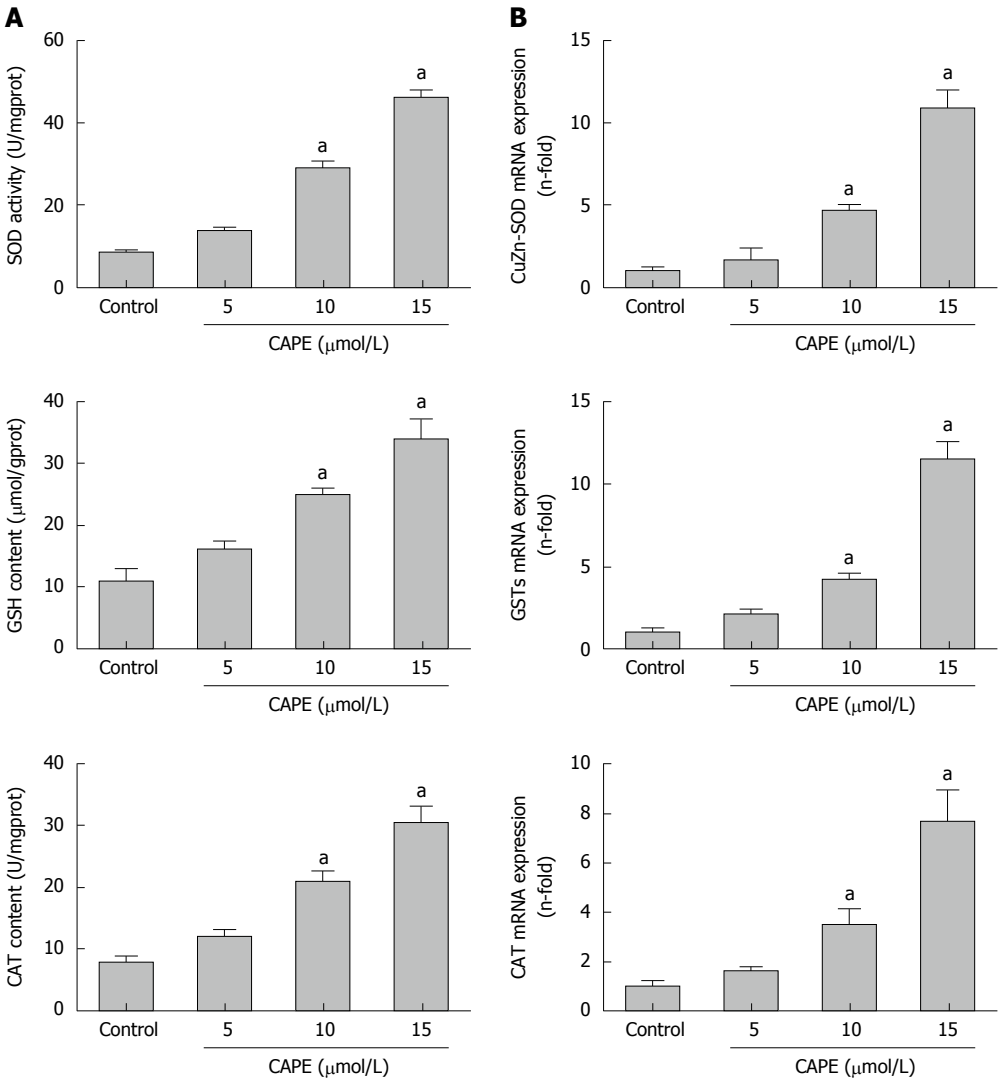

Figure 3 Antioxidant-related indicator protein and mRNA expression in hepatic stellate cell-T6 cells. After HSC-T6 cells were treated with 5 μmol/L, 10 μmol/L and 15 μmol/L CAPE for 24 h, the SOD activity, GSH and CAT content (A) and the mRNA expression of SOD, GSTs and CAT (B) were assessed. The data represent averages of the results of three independent experiments. [a]*P* < 0.05 *vs* control. CAPE: Caffeic acid phenethyl ester; SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-transferase.

and JNK1/2 were significantly increased in a dose-dependent manner in HSC-T6 cells after treatment with CAPE (5, 10 and 15 μmol/L) for 24 h compared to the control group ($P < 0.05$, Figure 5A). To further investigate the connection between Nrf2 and MAPKs pathways in HSC-T6 cells, HSC-T6 cells were pre-treated with the ERK1/2 inhibitor PD98059, the p38MAPK inhibitor SB203580, and the JNK1/2 inhibitor SP600125 for 2 h, followed by treatment with CAPE (15 μmol/L) for 24 h. All of the inhibitors partially decreased Nrf2 mRNA expression and nuclear/cytosol protein levels in HSC-T6 cells ($P < 0.05$, Figure 5B), suggesting that inhibition of MAPKs suppresses the translocation of Nrf2 protein in HSC-T6 cells.

Inhibitors of MAPKs and CAPE alter antioxidant-related indicator protein and mRNA expression in HSC-T6 cells

Gene and protein expression of SOD, CAT and GST was significantly decreased in HSC-T6 cells after incubation with the inhibitors of MAPKs and 15 μmol/L CAPE compared to the control group ($P < 0.05$, Figure 6A and B).

DISCUSSION

Previous studies have confirmed that CAPE has active biological antioxidant and anti-fibrosis properties[5,6,19,20]. Considering the fact that HSC proliferation and activation are key during liver fibrosis[21-23], it is interesting to know whether CAPE treatment can influence HSC proliferation and activation. Unfortunately, only one study has shown that CAPE inhibited HSC cell proliferation *in vitro*[24]. In this study, our data showed that the proliferation and activation of HSC-T6 cells were significantly inhibited and apoptosis was induced by CAPE in a dose-dependent manner. In addition, the expression of α-SMA and collegen-1 proteins was significantly reduced in HSC-T6 cells in response to CAPE. Taken together, these results suggested that CAPE inhibited

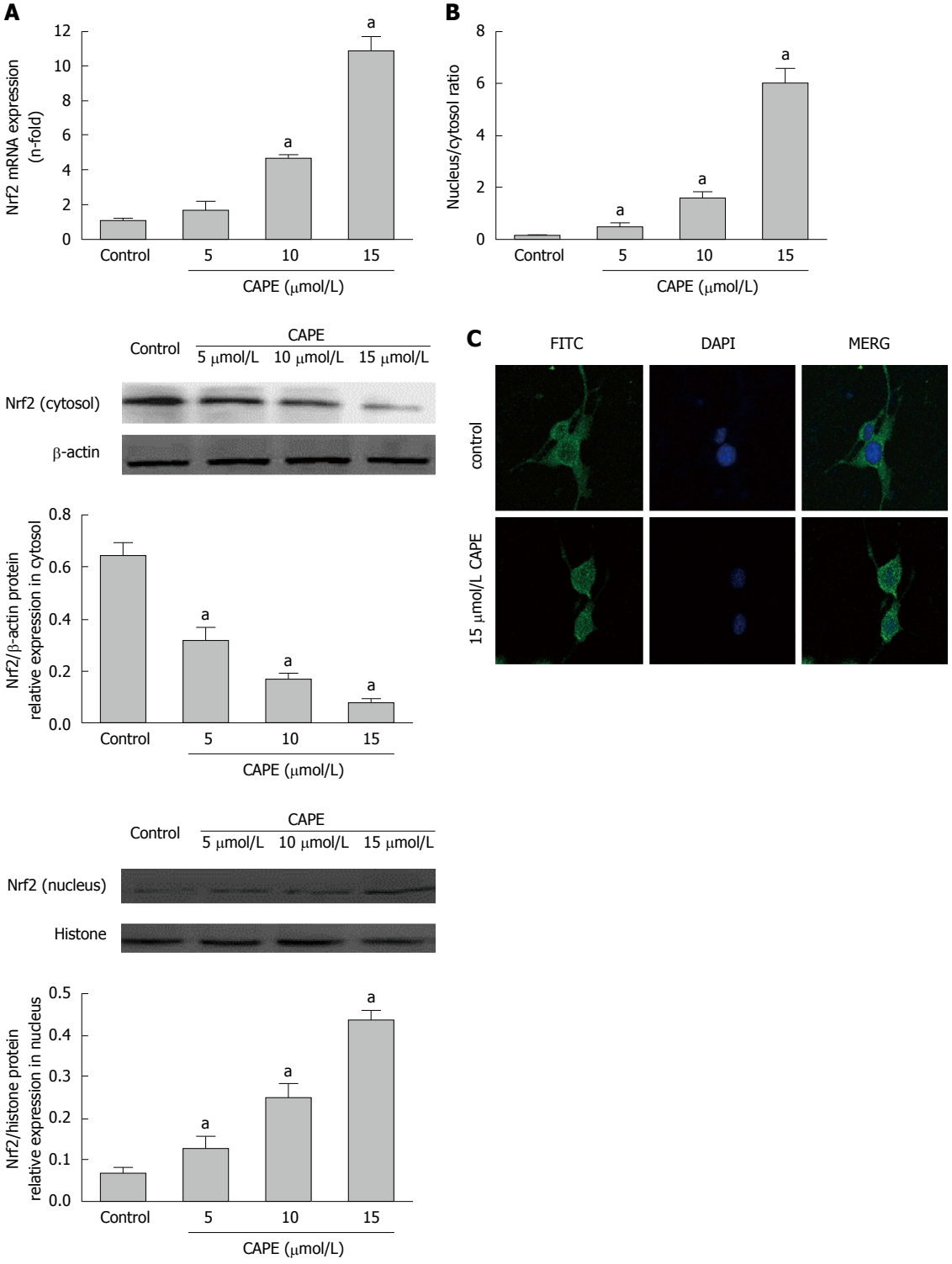

Figure 4 Effect of caffeic acid phenethyl ester on Nrf2 expression in hepatic stellate cell-T6 cells. A: HSC-T6 cells were treated with 5 μmol/L, 10 μmol/L and 15 μmol/L CAPE for 24 h. Nrf2 mRNA and cytosol and nuclear protein expression levels were investigated using real-time PCR and Western blot, respectively; B: The nucleus/cytosol ratio defines Nrf2 protein expression in the nucleus/Nrf2 protein expression in cytosol; C: Indirect immunofluorescence (× 200) analysis of Nrf2 protein expression in HSC-T6 cells. The upper: control group and the lower: 15 μmol/L CAPE group. The data presented are the mean ± SD (n = 3). $^{a}P < 0.05$ *vs* control. CAPE: Caffeic acid phenethyl ester.

HSC-T6 cell proliferation and activation, which is one of the key events initiating the occurrence and development of liver fibrosis.

CAPE has been confirmed to inhibit liver fibrosis in rats[8], in this study the results showed that CAPE increased the expression levels of SOD, CAT and GSH activities in HSC-T6 cells. Therefore, it is reasonable to suspect that CAPE may inhibit HSC cell proliferation and activation through its antioxidant effect. To confirm our hypothesis, the effect of CAPE on the expression of Nrf2, an upstream transcription factor regulating the expression of anti-oxidative stress factors, in HSC-T6

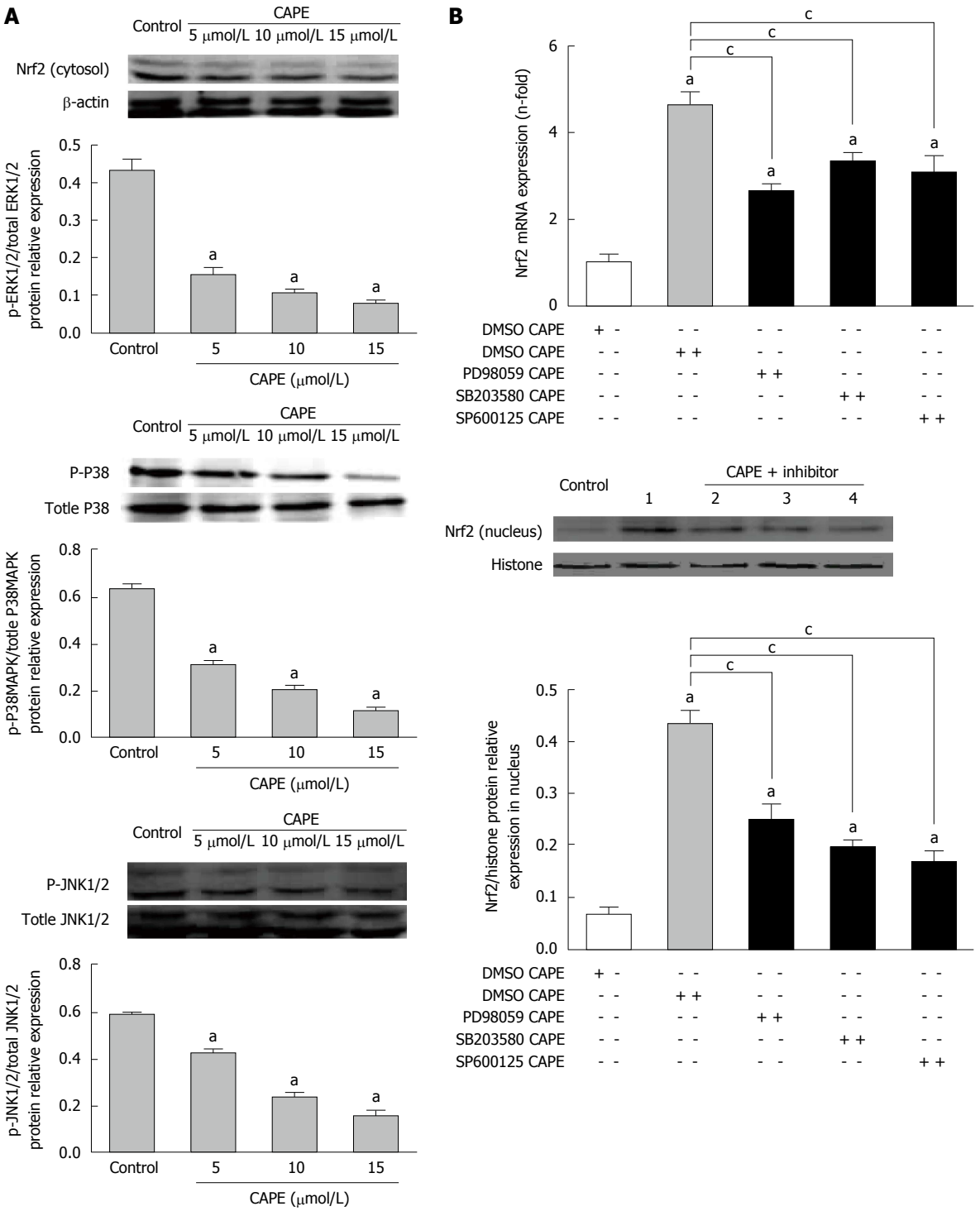

Figure 5 Phosphorylation levels of ERK1/2, p38MARK, and JNK1/2 are significantly increased in a dose-dependent manner in hepatic stellate cell-T6 cells. A: Effect of caffeic acid phenethyl ester on phosphorylation of ERK1/2, p38MAPK and JNK. Western blot analysis of total and phosphorylated protein levels of ERK1/2, p38MARK and JNK in HSC-T6 cells treated with CAPE (5 μmol/L, 10 μmol/L and 15 μmol/L) was performed. Total MAPKs were used as the internal control. The gray levels were normalized against those of the corresponding total MAPKs and the results are expressed, relative to control; B: Effect of inhibitors of MAPKs on the expression of Nrf2 in HSC-T6 cells. Cells were treated with ERK1/2 inhibitor PD98059 (30 μmol/L), p38 MAPK inhibitor SB203580 (20 μmol/L) or JNK inhibitor SP600125 (25 μmol/L) for 2 h, then incubated with CAPE (15 μmol/L) for 24 h, and protein expression was evaluated by Western blot. Lane 1: Control group; Lane 2: 15 μmol/L CAPE group; Lane 3: CAPE + PD98059 group; Lane 4: CAPE + SB203580 group; Lane 5: CAPE + SP600125 group. Histone was used as the internal control to reflect the expression of nuclear Nrf2. The gray levels were normalized against histone and the results are expressed, relative to control. The data are the mean ± SD of three independent experiments. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* CAPE group. CAPE: Caffeic acid phenethyl ester.

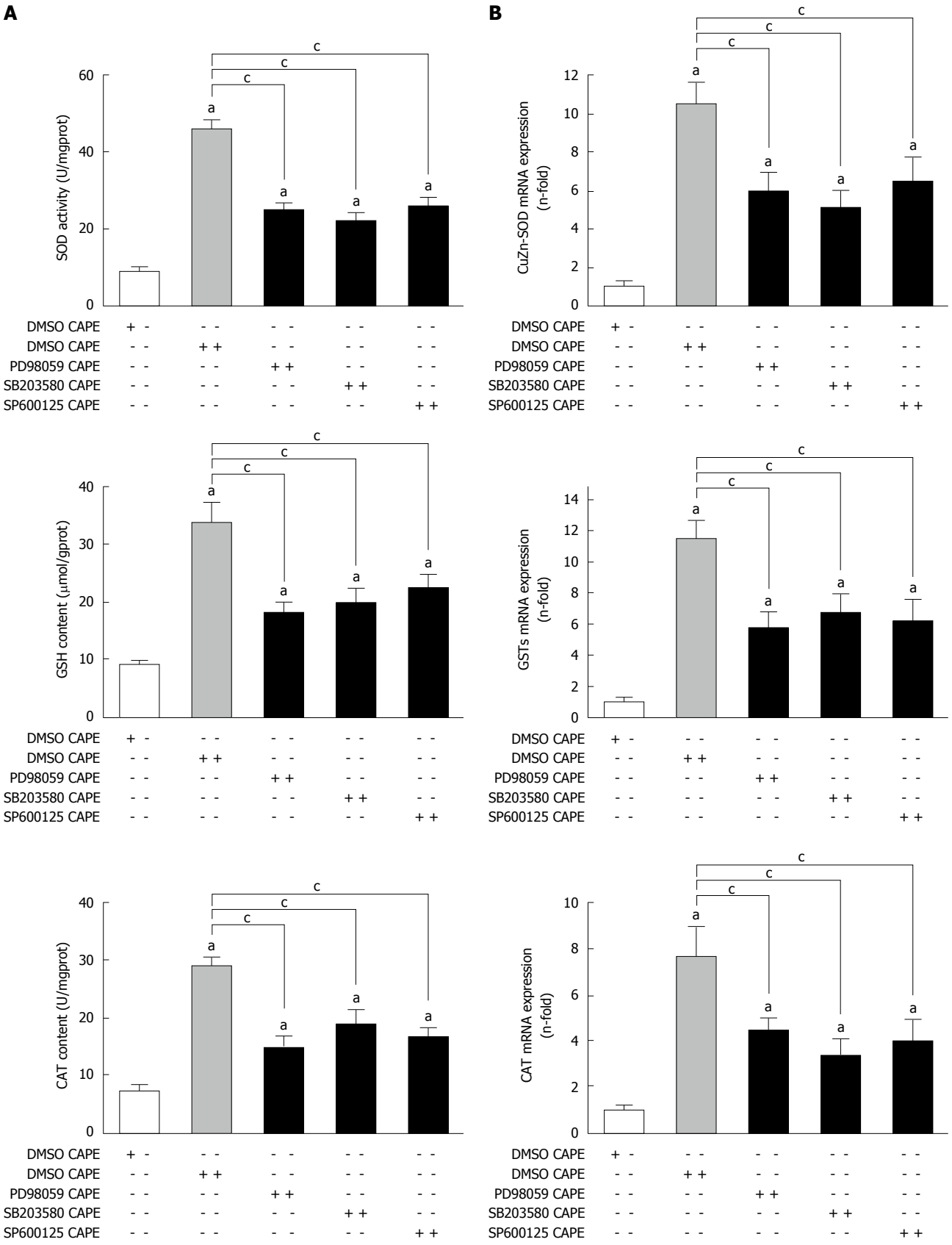

Figure 6 Effect of inhibitors of mitogen activated protein kinases and caffeic acid phenethyl ester on the antioxidant-related indicator protein and mRNA expression. A: Effect of inhibitors of MAPKs and CAPE on the SOD activity and GSH and CAT content in HSC-T6 cells. Cells were treated with the ERK1/2 inhibitor PD98059 (30 μmol/L), p38MAPK inhibitor SB203580 (20 μmol/L) or JNK inhibitor SP600125 (25 μmol/L) for 2 h, then incubated with CAPE (15 μmol/L) for 24 h; B: Effect of inhibitors of MAPKs and CAPE on antioxidant-related mRNA expression in HSC-T6 cells. The data represent averages of the results of three independent experiments. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* CAPE group. CAPE: Caffeic acid phenethyl ester; SOD: Superoxide dismutase; CAT: Catalase; GST: Glutathione-S-transferase.

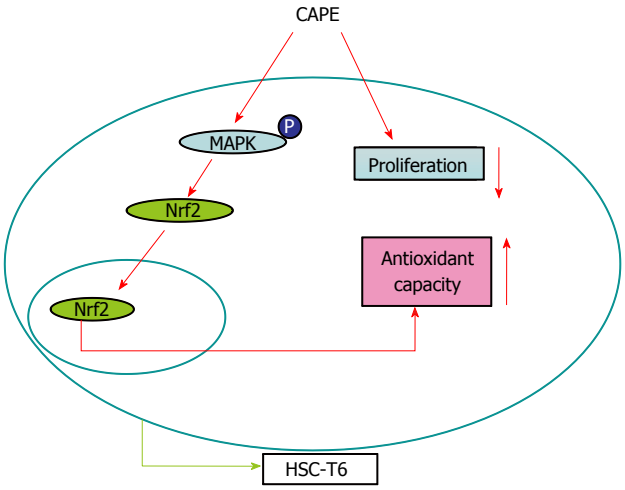

Figure 7 Nrf2-mediated mitogen activated protein kinases signaling pathway contributes to antioxidant capacity of caffeic acid phenethyl ester in hepatic stellate cell-T6 cells.

cells was investigated. Interestingly, we found that CAPE significantly increased *Nrf2* gene expression in HSC-T6 cells. Moreover, CAPE treatment significantly promoted Nrf2 protein translocation from the cytosol to the nucleus in HSC-T6 cells. These results indicated that CAPE promotes the synthesis and activation of Nrf2 in HSC cells.

The precise mechanism by which CAPE regulates Nrf2 expression and activation in HSC cells is still unknown. However, previous studies have shown that MAPKs, including ERK, JNK, and p38, play a role in regulating Nrf2 expression[25-30]. Therefore, we investigated the effects of CAPE on MAPKs signaling pathways in HSC-T6 cells. Our study showed that the phosphorylation levels of ERK1/2, p38MARK and JNK1/2 were significantly increased in a dose-dependent manner in HSC-T6 cells after treatment with CAPE. Meanwhile, pretreatment of HSC-T6 cells with inhibitors significantly attenuated antioxidant enzyme expression. Moreover, all of the inhibitors partially decreased Nrf2 mRNA expression and nuclear/cytosol protein levels in HSC-T6 cells, suggesting that inhibition of MAPKs suppresses the translocation of Nrf2 protein in HSC-T6 cells. Taken together, our results are the first report that MAPKs are the key signaling pathways involved in the CAPE-induced anti-oxidative responses in cultured HSC-T6 cells.

In conclusion, we demonstrated that CAPE inhibited cell proliferation, induced cell apoptosis, increased expression levels of SOD, CAT and GSH, and decreased α-SMA expression level in HSC-T6 cells by activation of the Nrf2-mediated MAPKs signaling pathways. We propose a working model of the antioxidative role and potential mechanism of CAPE in HSC-T6 cells (Figure 7). In this model, CAPE inhibits cell proliferation and activation and increases the expression levels of antioxidative enzymes in HSC-T6 cells, which might partly depend on an Nrf2-mediated MAPKs signaling pathway, and thereby is a potential anti-fibrosis agent for the treatment of human liver fibrosis.

COMMENTS

Background

Liver fibrosis is a pathological response to hepatocyte injury, including oxidative stress, which is a primary mechanism of liver damage. Oxidative stress can stimulate the activation of hepatic stellate cells (HSCs) through paracrine and autocrine, leading to the formation of liver fibrosis. Antioxidants play a pivotal role in the development of liver fibrosis. Caffeic acid phenethyl ester (CAPE) is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. However, it is still largely unknown whether CAPE can reduce the expression of these factors *via* the Nrf2-mediated mitogen activated protein kinases (MAPKs) signaling pathway in HSC.

Research frontiers

Previous studies proved that CAPE has active biological antioxidant and anti-fibrosis properties, also their previous studies have shown that CAPE inhibited liver fibrosis in rats.

Innovations and breakthroughs

In this study, the authors demonstrated that CAPE inhibited cell proliferation, induced cell apoptosis, increased expression levels of SOD, CAT and GSH, and decreased α-SMA expression level in HSC-T6 cells by activation of the Nrf2-mediated MAPKs signaling pathways. They propose a working model of the anti-oxidative role and potential mechanism of CAPE in HSC-T6 cells.

Applications

CAPE is a phenolic compound extracted from honeybee propolis that has strong biological properties in liver protection and as an antioxidant and anti-fibrosis agent. It has been used in the treatment of several diseases. These data also indicate that CAPE can up-regulate the antioxidant levels in HSC-T6 cells partly through the Nrf2-MAPKs signaling pathway and thereby is a potential anti-fibrosis agent for the treatment of human liver fibrosis in the future.

Terminology

MAPK: a type of protein kinase that is specific to the amino acids serine, threonine, and tyrosine (*i.e.*, a serine/threonine-specific protein kinase). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock, and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. CAPE: a phenolic compound extracted from honeybee propolis.

Peer-review

This is an interesting study showing that caffeic acid phenethyl ester up-regulates antioxidant levels in the hepatic stellate cell line. In this study, the authors investigated the antioxidation effect of caffeic acid phenethyl ester in hepatic stellate cells T6 cultured *in vitro* and the potential mechanisms of the effect. The effect on a-smooth muscle actin was shown using immunofluorescence. Gene and protein levels of Nrf2, including related factors and mitogen activated protein kinases, in HSC-T6 cells were detected using RT-PCR and Western blot, respectively. CAPE inhibited the proliferation and activation of HSC-T6 cells cultured in vitro. CAPE increased the antioxidant levels and the translocation of Nrf2 from the cytoplasm to the nucleus in HSC-T6 cells. Moreover, the phosphorylation of MAPKs in cells decreased in response to CAPE. Interestingly, CAPE-induced oxidative stress in the cells was significantly attenuated by pretreatment with MAPKs inhibitors. Overall, this study is well designed, and the results are interesting.

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