Effect of caffeic acid phenethyl ester on proliferation and apoptosis of hepatic stellate cells in vitro

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INTRODUCTION

Liver fibrosis is a progressive pathological process involving multi-cellular and molecular events that ultimately lead to deposition of excess matrix proteins in the extracellular space. It is generally accepted that HSC is the most pathogenetically relevant cell type for the development of liver fibrosis[3-11]. During liver injury and inflammation, quiescent HSCs transdifferentiate into activated-type HSCs, also termed myofibroblasts (MFBs), which result in accumulation of a cell type that provides main collagenous and non-collagenous components of the extracellular fibrotic matrix, contribute to diminished degradation of extracellular matrix by the expression of tissue inhibitors of metalloproteinases (TIMPs) and also produce an array of proinflammatory cytokines and chemokines involving the development of liver fibrosis[3-11].

It has recently been shown that the recovery from established experimental fibrosis can occur through the apoptosis of HSC, which is associated with reduction of collagen and expression of TIMPs in liver. Apoptosis, therefore, plays an important role in the resolution of fibrosis by eliminating the sources of both the neomatrix and TIMPs, and thereby facilitates net matrix degradation[3-11]. Activation and apoptosis of HSCs could play a central role in turnover of liver fibrosis.

Recent research showed that cultured HSC underwent a rapid and persistent induction of a high-mobility NF-κB DNA binding complex, and the activation of NF-κB in cultured HSC was required for activated phenotype of HSCs and might be anti-apoptotic for HSC[16-19]. In this study, we investigated the effect of NF-κB inhibitor CAPE on the proliferation, collagen synthesis and apoptosis of HSCs.

MATERIALS AND METHODS

Materials

Wistar rats, male, 450-500 g of body mass, were provided by the Center for Laboratory Animal of Kunming General Hospital. DMEM and FBS were obtained from GIBCO. CAPE and collagenase were purchased from SIGMA. "H-TdR and "H-proline were provided by Beijing Institute of Atomic Energy. RNA Mimi Kit and DIG DNA Labeling and Detecting Kit were respectively obtained from Gene and Boehringer Mannhelm.

Methods

Isolation and culture of HSCs

HSCs were isolated from normal liver by sequential in situ perfusion with collagenase, as previously described[20-21]. Briefly, the livers were perfused first with Ca²⁺- and mg²⁺-free solution for 10 min at 37 °C, and next with 0.05 % (w/v) collagenase solution for 30 min at 37 °C. The digest liver were excised, dispersed in D-Hanks, and filtered through gauze. The residual hepatocytes were removed by two low-speed centrifugation (50 g, 4 °C 2 min). The stellate-cell enriched fraction was obtained by centrifugation with a triple-layered (9, 11, and 17 %) Nycodenz cushion (1 400×g, 4 °C 2 min). The cells in the upper-layer were washed and seeded onto uncoated plastic tissue culture plate in DMEM supplemented with 10 % FBS and grown for 14 days. Purity and viability of freshly isolated HSC were determined by Desmin immunohistochemistry and trypan-blue stain respectively.

"H-TdR or "H-proline uptake by HSC

Passaged HSCs were cultured in 24-well plates for 48 h and then treated with CAPE at concentrations of 0.0, 1.0, 2.5, 5.0, 10.0, 20.0, and 40.0 µmol/ L for 24 h. CAPE was dissolved in DMSO. The cells were then labeled with 1 µci/ml "H-TdR or 1 µci/ml "H-proline.
respectively. After washed with D-Hanks, the HSCs were digested with trypsin and absorbed on glass filter paper. The cells were then washed once with 10 % trichloroacetic acid and three times with saline and dried overnight at 80 °C. The radioactivity (CPM) of each sample was counted using liquid-scintillation analyzer.

Expression of procollagen gene in HSC Type I and III procollagen gene sequences were enquired from GeneBank of National Center of Biotechnology Information, and primers that amplified the procollagen genes were designed with OLIGO microsoft according to the gene sequences and synthesized by Sangon Company. The primers were 5’-CGA TGG ATT CCC GTT CGA GTA C-3’ and 5’-GTC CAC AAC CCT GTA GGT G-3’ for type I procollagen gene, 5’-GGA AAC AGC AAA TTC ACT TAC A-3’ and 5’-TCA CTT GCA CTG GTT GAT AAG A-3’ for type III procollagen gene. The RNA was extracted from the skin of newborn ICR mice by RNA mini kit, the genes were amplified by RT-PCR and procollagen gene probes were labeled with PCR and DIG-dUTP. HSCs cultured in 24-well plates were treated with 20 µmol/L CAPE for 24 h and the procollagen gene expression was detected according to the previously described procedure[22].

Apoptosis assay Passaged HSCs were cultured for 48 h and then cultured in 5 % FBS DMEM and treated with CAPE at predetermined concentrations of 0.0, 20.0, and 40.0 µmol/L for 12 h and 24 h, the cells were fixed in formaldehyde solution and stained with TUNEL reaction solution containing DIG-dUTP and terminal deoxynucleotide transferase for 60 min at 37 °C. DIG was detected by anti-DIG-AP conjugate and colorimetric substrate NBT/BCIT. Positive cells were counted under microscope.

RESULTS
Characteristics of HSCs
Freshly isolated HSCs were round-shaped with many yellow-coloured droplets in cytoplasm, after 2-3 days in culture on uncoated plastic surface, the cells had spread and showed a typical ‘star’-like configuration. More than 80 % of freshly isolated cells were desmin-positive, and the cell viability was about 90 % according to trypan-blue staining.

Effect of CAPE on 3H-TdR and 3H-proline incorporation by HSC
We observed the effect of CAPE on 3H-TdR and 3H-proline incorporation by cultured HSCs. As shown in Figure 2, CAPE significantly and dose-dependently suppressed the incorporation of 3H-TdR and 3H-proline by HSCs. The median inhibitory concentrations were 5 and 10 µmol/L respectively for 3H-TdR and 3H-proline. These concentrations were lower, especially for the inhibition of HSCs proliferation, indicating that CAPE was a potent inhibitor for proliferation, and collagen synthesis.

Figure 2 Effects of CAPE on 3H-TdR and 3H-proline uptake by HSCs. P<0.01 vs groups without CAPE treatment.

Figure 3 Effect of CAPE on type I and III procollagen gene expression. P<0.01 vs the group without CAPE treatment.

Figure 4 Apoptotic HSCs detected by TUNEL without (A) or with 40 µmol/L CAPE treatment (B).

Effects of CAPE on procollagen gene expression
Type I and III collagens are the main components of extracellular matrix in liver fibrosis. In situ hybridization
analysis of type I and III procollagen genes showed that positive HSCs were reduced by CAPE at the concentration of 20 μmol/L. And the reduction of type I procollagen gene expression was statistically significant, indicating that CAPE suppressed the procollagen gene expression.

**Apoptosis of HSC induced by CAPE**

Apoptosis of HSC was demonstrated by TUNEL, the nucleus of apoptotic HSCs were stained with violet blue (Figure 4). After treatment with CAPE, AIs were time- and dose-dependently increased from 2.82±0.73 % to 7.66±1.25 % at 12h and from 3.15±0.88 % to 10.61±2.88 % at 24 h. The data indicated that CAPE induced HSC apoptosis at higher concentrations (Figure 5).

**Figure 5 Effects of CAPE on HSC apoptosis. P<0.05; P<0.01 vs the groups without CAPE treatment; P<0.01 vs the group with 40 μmol/L CAPE treated for 12 h.**

**DISCUSSION**

HSCs, previously termed as fat or vitamin A-storing cells or Ito cells, localized in close proximity to sinusoidal endothelial cell and hepatocyte in the space of Disse, are the most pathogenetically relevant cell type for development of liver fibrosis[23]. Activated HSCs in liver tissue provide virtually most main components of ECM, contribute to diminished degradation of ECM by expressing TIMPs, and produce an array of proinflammatory cytokines and chemokines involving the development of liver fibrosis[3-11]. It is generally accepted that HSCs are important target cells for the treatment of liver fibrosis[12-13]. In our study, we observed that the NF-κB inhibitor CAPE inhibited the proliferation and collagen synthesis of HSCs. CAPE dose-dependently suppressed the incorporation of 3H-TdR and 3H-proline by HSCs. This indicated CAPE inhibited the proliferation of HSCs. Because the reduction of either total cell number or collagen synthesis may contribute to the reduction of 3H-proline incorporation by HSCs, the expressions of type I and type III procollagen genes were further explored in HSCs. Our data showed that the procollagen gene expressions were reduced by CAPE, and the reduction of 3H-proline uptake partially indicated that CAPE induced collagen synthesis, in addition to the reduction of HSC number.

CAPE, a structural relative of flavonoids that is an active component of propolis from honeybee hives, has been shown to inhibit the growth of different types of cells including endothelial cells, keratinocyte and tumor cells, which involves in nuclear factor, protein kinase C and cytokine signal transduction[24-29]. CAPE inhibited the proliferation and collagen synthesis of HSCs, properly by reducing the reactive oxygen intermediates, this is consistent with the antioxidant property of CAPE[30,31]. Previous studies showed that reactive oxidant species induced HSCs activation and collagen gene expression in vivo and in vitro[22-23]. The antioxidant phenolic compounds reduced the proliferation and collagen synthesis by suppressing inositol phosphate metabolism, tyrosine and protein kinase activation[33,36]. Since CAPE is a structural relative of flavonoids, CAPE probably inhibits the proliferation and collagen synthesis of HSCs similarly. Other mechanisms might include inhibition of the expression of COX2 and activation of NF-κB by CAPE, which are associated with the phenotype of activated HSCs[37,38].

Activated HSCs undergo auto-apoptosis in serum-deprived DMEM and in experimental fibrosis induced by CCL[27] and Fas have already been identified as molecules associated with the apoptosis of HSCs, which were expressed in HSCs. Apoptosis of HSCs can be induced by nerve growth factor and soluble Fas-ligand[39,40]. The recovery from established experimental fibrosis is relevant to the apoptosis of HSCs, which contributes to the reduction of neomatrix synthesis and expression of TIMPs. Recent reports showed the persistent activation of NF-κB was induced in activated but not in quiescent HSCs, which is required for activated phenotype of HSCs and may be antiapoptotic for HSCs[16-18]. CAPE is a potent and specific inhibitor of NF-κB, CAPE inhibits the activation of NF-κB induced by TNF-α and other inflammatory agents including phorbol ester, ceramide, hydrogen peroxide, etc[41]. Our data showed the AIs of HSCs were increased by CAPE, the inhibition of NF-κB activation by CAPE probably played a key role in HSC apoptosis. CAPE inhibited the activation of NF-κB and the expression of proinflammatory cytokines TNF-α and IL-1β; CAPE also induced apoptosis in macrophages[42]. Similar results were observed in leucocytes as well[43]. However, CAPE induced apoptosis of tumor cells by regulating the expression of caspases-3, -bcl-2, bax and P[34,46]. For HSCs, the mechanism of apoptosis induced by CAPE needs further investigation.

CAPE has been shown to be a pharmacologically safe compound with known antiinflammatory, antimitogenic, anticarcinogenic, antioxidant, and immunomodulatory effects[35-67]. Therefore, CAPE might have a therapeutic role in liver fibrosis by inhibiting the proliferation or inducing the apoptosis of HSCs.

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Edited by Yuan HT