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Butein suppresses myofibroblastic differentiation of rat hepatic stellate cells in primary culture

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Abstract

Hepatic stellate cells play a key role in the pathogenesis of hepatic fibrosis. In this study, we investigate the inhibitory effect of butein on the activation and proliferation of rat primary cultured hepatic stellate cells. Possible cytotoxic effects were measured on stellate cells and hepatocytes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of butein on the production of collagen and smooth muscle α-actin proteins were examined at the same concentration, by western blot. The effects of butein on α1(I) collagen, tissue inhibitor of metalloproteinase-1, and metalloproteinase-13 gene expression in activated stellate cells were investigated by measuring mRNA levels using reverse transcription polymerase chain reaction. The effect of butein on DNA synthesis was also determined. Butein, at a concentration of 1 μg mL^{-1}, reduced DNA synthesis without affecting cell viability, and downregulated smooth muscle α-actin and type-I collagen expression, and α1(I) collagen and tissue inhibitor of metalloproteinase-1 mRNA expression, while treatment with butein induced metalloproteinase-13 mRNA expression. These findings suggest that butein is a potent inhibitor of stellate cell transformation.

Introduction

Hepatic stellate cells (HSCs) have been postulated to play a critical role in the development of hepatic fibrosis (Friedman 1993). Hepatic fibrosis is a common response to chronic liver disease of various aetiologies, such as persistent viral hepatitis, alcohol overload or autoimmune liver disease. It is caused by changes in both the amount and the composition of the extracellular matrix. Steady-state levels of extracellular-matrix proteins are regulated by their rates of synthesis and degradation. Current evidence suggests that stellate cells are central to this process, as the major source of collagens, matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) (Rojkind et al 1979; Gressner & Bachem 1990; Friedman 1993). Normally these cells are liver-specific pericytes that serve as major vitamin A storage sites and show little proliferative activity. In the injured liver, however, stellate cells transform to a myofibroblast-like phenotype, a process termed activation. During this phenotypic transition, they lose their vitamin-A-containing fat droplets and acquire the capacity to proliferate and express receptors for various growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) (Pinzani et al 1989; Ramadori 1991; Gressner 1995). In addition, activated HSCs express smooth muscle cell α-actin (α-SMA), collagens and other matrix proteins that are deposited during fibrosis. Accumulation of extracellular matrix, therefore, occurs from an increase in the numbers of HSCs as well as the synthesis and secretion of matrix proteins when cells are in the activated phenotype (Friedman et al 1985; Knittel et al 1992; Friedman 2000). Given the pivotal role of activated HSCs in chronic liver injury, agents that could regulate HSC activation may be promising candidates as therapeutic agents against collagen deposition in chronic liver injury (Friedman 1993).

It has been reported that some naturally occurring products inhibit the activation of cultured stellate cells. In this context, vitamin E (Lee et al 1995), quercetin (Kawada et al 1998), baicalein (Inoue & Jackson 1999), tetrandrine (Park et al 2000)

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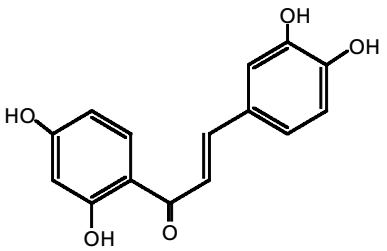

Figure 1 Chemical structure of butein, 3,4,2′,4′-tetrahydroxychalcone.

and curcumin (Kang et al 2002), have all been shown to inhibit the activation of cultured stellate cells, as well-known antioxidants. Oxidative stress enhances the proliferation of cultured stellate cells and their subsequent collagen synthesis (Casini et al 1994), whereas the presence of antioxidants may suppress this process.

Butein (3,4,2′,4′-tetrahydroxychalcone) (Figure 1), a plant polyphenol, is a chalcone compound belonging to the flavonoid subclass. Butein has a structure similar to curcumin, and has been shown to possess antioxidant effects (Sogawa et al 1994; Zhang et al 1997; Cheng et al 1998). For several years, we have been screening candidate antifibrotics from natural compounds, and, as stated above, butein has an antioxidant activity, although direct evidence to support its effect on activated HSCs is not yet available. In this study, the effects of butein on myofibroblastic differentiation were investigated in isolated HSCs, activated in-vitro, by determining DNA synthesis, the expression of collagen and α-SMA based on protein levels, and the levels of mRNA for α1(I) collagen, tissue inhibitor of metalloproteinase-1 (TIMP-1) and metalloproteinase-13 (MMP-13).

Materials and Methods

Butein solution

Butein was purchased from Calbiochem-Novabiochem (La Jolla, CA). It was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) at 10 mg mL^{-1}, diluted and added to the culture medium to give a final concentration of 0.1% (v/v) DMSO.

Isolation and cultivation of HSCs

Hepatic stellate cells were isolated from the livers of untreated normal male Sprague-Dawley rats weighing 450–500 g by two-step collagenase perfusion (Woo et al 2002). Isolated cells were more than 90% viable as assessed by trypan blue exclusion, and consisted of more than 90% hepatic stellate cells as determined by immunostaining with horseradish-peroxidase-coupled anti-desmin (Sigma).

Cell viability test

Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. HSCs were cultured in 96-well plates and grown to confluence in William's Medium E (WME, Gibco BRL, USA) containing 10% fetal bovine serum (FBS, Gibco BRL) under standard culture conditions for five days. HSCs were then incubated in WME containing 0.5% FBS for 24 h. Thereafter, the medium was removed and the cells were then incubated for an additional 24 or 48 h in the presence of various concentrations of butein (0.1–10 μg mL^{-1}). MTT (1 mg mL^{-1}) was added for the last four hours of the incubation. Reduction of MTT to formazan was assessed in an ELISA plate reader (Park et al 2000).

Measurement of DNA synthesis

DNA synthesis was measured by indirect immunoperoxidase staining of nuclei that had incorporated bromodeoxyuridine (BrdU) using an ELISA kit (Roche Molecular Biochemicals) (Park et al 2000). Briefly, to investigate BrdU incorporation, HSCs were cultured in 96-well plates and grown to confluence in WME medium containing 10% FBS under standard culture conditions for five days. HSCs were then incubated in WME containing 0.5% FBS for 24 h. Thereafter, the medium was removed and the cells were then incubated for a further 24 h with 40 ng mL^{-1} platelet-derived growth factor-BB (PDGF-BB) (Pinzani et al 1989), or 50 μmol L^{-1} Fe^{2+} per 100 μmol L^{-1} ascorbate (FeAsc) (Parola et al 1993; Svegliati-Baroni et al 2001), in the presence or absence of different concentrations of butein. They were then incubated with BrdU for another 24 h. After removing the culture medium, the cells were fixed, incubated with anti-BrdU peroxidase and the incorporated BrdU was detected by the subsequent substrate reaction according to the manufacturer's advice.

Western blot analysis

Primary stellate cells were cultured at a density of 5×10^5 cells/mL on uncoated plastic dishes and grown to confluence in WME medium containing 10% FBS under standard culture conditions for five days. HSCs were then incubated in WME containing 0.5% FBS for 24 h. Thereafter, the medium was removed and the cells then incubated for a further 24 h or 48 h, in the presence or absence of different concentrations of butein. Collagen was examined in the medium and α-SMA was examined in the cell lysate. Cells were then washed twice with Hank's balanced salt solution and lysed directly in RIPA buffer (50 mmol L^{-1} Tris-HCl pH 7.4, 1% (v/v) Triton X-100, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} leupeptin, 1 mmol L^{-1} phenylmethylsulfonyl fluoride). HSCs were centrifuged at 14 000 rev min^{-1} for 30 min at 4 °C and the supernatant collected. The medium and protein was concentrated using Centriplus tubes (Millipore, USA) to retain molecules over 30 kDa molecular weight. Protein concentration was then determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, USA). Extracts from the cell lysates (20 μg) or medium (20 μg) were separated by 10% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were treated with a blocking buffer comprising

5% (v/v) non-fat milk in phosphate-buffered saline (PBS) and then incubated with monoclonal anti-α-SMA antibody (Sigma; diluted 1/250) or rabbit anti-collagen type I antibody (Calbiochem; diluted 1/200) for two hours at room temperature. After vigorous washing, the membrane was then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Santacruz, USA; diluted 1/2000) for one hour at room temperature. Immunoreactive bands were visualized on X-ray film (Agfa, EC) using the enhanced chemiluminescence western blotting detection system kit (Amersham, USA) according to the manufacturer's recommended protocol. The developed films were subjected to densitometric analysis (JX-330P, Pharmacia, USA).

Quantification of mRNA level by reverse transcription polymerase chain reaction (RT-PCR)

Primary stellate cells were cultured at a density of 5×10^5 cells/mL on uncoated plastic dishes and grown to confluence in WME medium containing 10% FBS under standard culture conditions for five days. HSCs were then incubated in WME containing 0.5% FBS for 24 h. Thereafter, the medium was removed and the cells then incubated for a further 24 h, in the presence or absence of different concentrations of butein. Total RNA was extracted from the cells using an RNeasy kit (Qiagen, Germany). The concentration and purity of RNA were determined by spectrophotometry at 260/280 nm and the integrity of the RNA was verified by visualization of the 18S and 28S rRNA bands after agarose electrophoresis and ethidium bromide staining.

Five micrograms of total RNA was reverse-transcribed using oligo-dT (15–18-mer) primers and 200 U moloney murine leukaemia virus reverse transcriptase (Gibco BRL) at 42 °C, as described previously (Lee et al 2001). The sequences of primers used to detect specific α1(I) collagen, TIMP-1 and MMP-13 are given below. The PCR reaction was performed in the presence of 1.5 mM $MgCl_2$ at the following temperatures and times: 94 °C, 30s; 55–57 °C, 30s; 72 °C, 30s; with a final extension at 72 °C for 5 min. The levels of expression of all transcripts were normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) m RNA in the same tissue sample. Rat α1(I) collagen: sense 5′-TAC TAC CGG GCC GAT GAT G-3′; antisense 5′-CTT GGG GTT TGG GCT GAT GT-3′. Rat TIMP-1: sense 5′-CCA CAG ATA TCC GGT TCG CCT ACA-3′; antisense 5′-GAC CAC CCC ACA GCC AGC ACT AT-3′. Rat MMP-13: sense 5′-AAA GAA CAT GGT GAC TTC TAC C-3′; antisense 5′-ACT GGA TTC CTT GAA CGT C-3′. Amplified products were separated by electrophoresis on an agarose gel containing ethidium bromide and analysed for molecular size.

Statistical analysis

Results are expressed as means ± s.d. Statistical differences were determined between groups by non-parametric Kruskal-Wallis test and Dunns multiple comparison test. Statistically significant differences between groups were defined as having P values of less than 0.05. Calculations were performed with the Graphpad Prism program (Graphpad Software, Inc., San Diego, CA).

Ethical considerations

This experiment was carried out under the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. The animals care committee in our institution approved this study.

Results

Effect of butein on hepatic stellate cell viability

To determine any possible toxicity butein might have on HSCs, cell viability was assessed by the MTT assay. This showed that butein did not alter cell viability at the concentrations used in subsequent experiments (Figure 2).

Effect of butein on DNA synthesis with PDGF-BB and FeAsc stimulation

Butein markedly suppressed BrdU incorporation in a concentration-dependent manner in activated HSCs stimulated with FeAsc. BrdU incorporation in FeAsc-stimulated HSCs was 164 ± 4.2% of the unstimulated HSCs. It was significantly reduced to 112 ± 6.4% ($P < 0.05$) in FeAsc-stimulated HSCs treated with 1 μg mL^{-1} butein (Figure 3A). Butein also suppressed BrdU incorporation with PDGF-BB-stimulated HSCs in a concentration-dependent manner. BrdU incorporation in

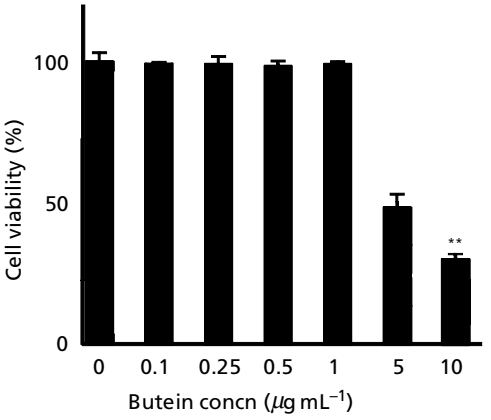

Figure 2 Effects of butein treatment on cell viability in rat hepatic stellate cells (HSC). HSC were exposed to butein at the indicated concentrations for two days. Cell viability was determined using 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. Results are the mean ± s.d. of three independent experiments, each performed using triplicate wells. $^{**}P < 0.05$, compared with HSC without butein.

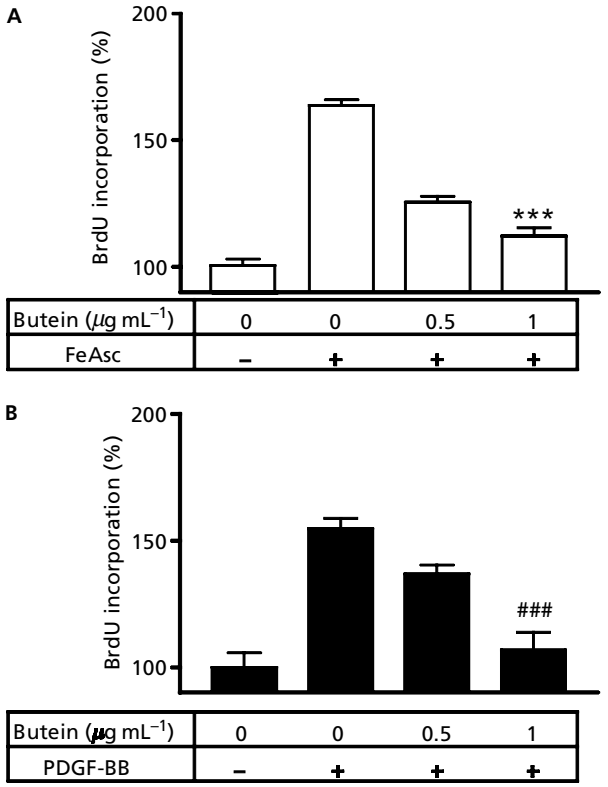

Figure 3 Effects of butein on DNA synthesis by rat cultured activated hepatic stellate cells stimulated with iron ascorbate (FeAsc) (A) or PDGF-BB (B). BrdU incorporation in cells was determined after incubation for 24 h with 50 μmol FeAsc, 40 ng mL^{-1} PDGF-BB and butein at concentration of 0, 0.5, and 1.0 μg mL^{-1}, as described in Materials and Methods. The control value without FeAsc or PDGF-BB was assumed as 100%. Results are the means ± s.d. of three independent experiments, each performed using triplicate wells. ***$P < 0.05$, compared with HSC treated with 50 μmol FeAsc alone; ###$P < 0.05$, compared with HSC treated with 40 ng mL^{-1} PDGF-BB alone.

PDGF-BB-stimulated HSCs was 154 ± 8% of the unstimulated HSCs, while it was significantly reduced to 107 ± 14% ($P < 0.05$) in PDGF-BB-stimulated HSCs treated with 1 μg mL^{-1} butein (Figure 3B).

Type-I collagen and α-SMA expression in butein treated HSCs

The inhibitory effect of butein on type-I collagen and α-SMA expression in activated HSCs cultured on plastic dishes was investigated by measuring protein levels using immunoblotting. When expressed as a percentage of activated HSCs treated with vehicle alone (given the arbitrary value of 100%), one day of butein treatment at concentrations of 0.5 and 1.0 μg mL^{-1} decreased type-I collagen to 67 ± 1% and 57 ± 5% ($P < 0.05$) and two days of butein treatment at concentrations of 0.5 and 1.0 μg mL^{-1} decreased type-I collagen to 53 ± 14% and 40 ± 13% ($P < 0.05$) in culture medium (Figure 4A), respectively, when compared with activated HSCs treated with vehicle alone.

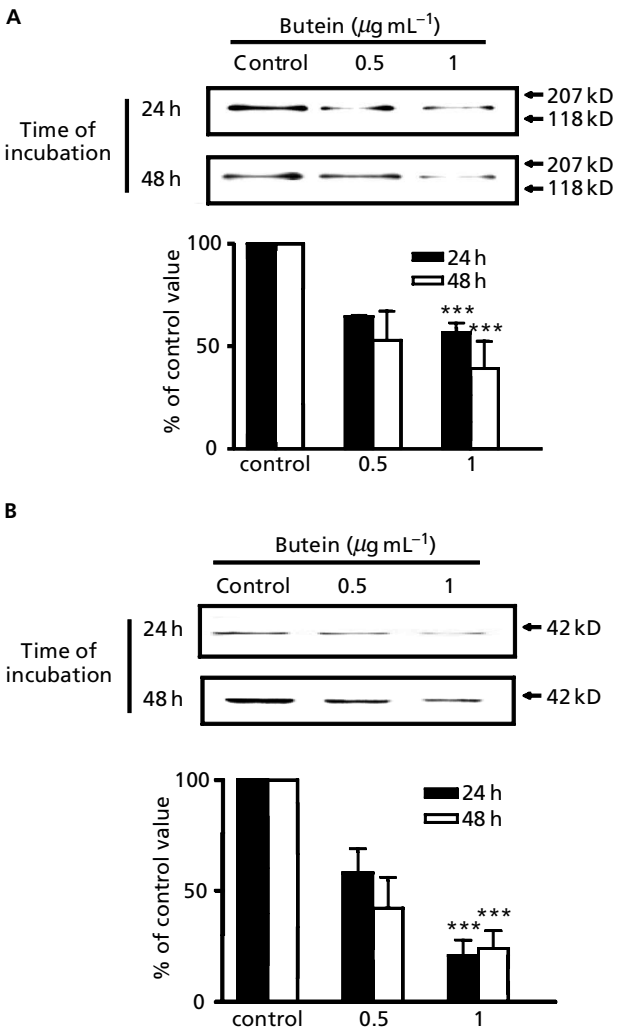

Figure 4 Western blot analysis for type-I collagen and smooth muscle α-actin expression in rat activated hepatic stellate cells. Cells cultured in WME for 5 days were incubated for additional 24 or 48 h in the absence or presence of butein at the indicated concentration. A. Type-I collagen secreted into culture medium. B. Smooth muscle α-actin in cell lysates. A representative feature of immunoreactive bands and the densitometric measurement (mean ± s.d., n = 3) are shown. ***$P < 0.05$, compared with HSC without butein.

Butein also suppressed the synthesis of α-SMA, an established marker for myofibroblastic differentiation. As shown in Figure 4B, the synthesis of this molecule was decreased to 58 ± 11% and 21 ± 7% ($P < 0.05$) of activated HSCs treated with vehicle alone by 0.5 and 1.0 μg mL^{-1} of butein for one day, respectively. The same concentrations of butein for two days decreased expression to 42 ± 14% and 24 ± 8% ($P < 0.05$) of activated HSCs treated with vehicle alone level, respectively. These experiments demonstrate increased α-SMA expression over time, and a reduction in the expression of this protein at all time points, during the culture of stellate cells in the presence of butein. The inhibition of α-SMA expression by butein was also shown to be dose dependent.

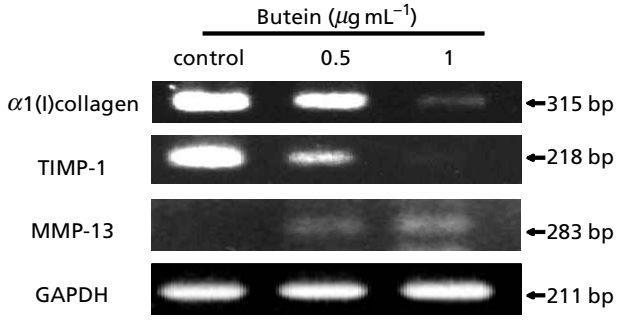

Figure 5 Transcripts of α1(I) collagen, tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-13 (MMP-13) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) were analysed by reverse transcriptase polymerase chain reaction (RT-PCR). Cells cultured in WME for 5 days were incubated for additional one day in the absence or presence of the indicated concentration. Total RNA was purified from rat activated hepatic stellate cells with or without butein at the indicated concentration.

Quantification of mRNA level by reverse transcription polymerase chain reaction

The inhibitory effect of butein on α1(I) collagen and TIMP-1 gene expression in activated HSCs cultured on plastic dishes was investigated by measuring mRNA levels using RT-PCR.

Butein induced a significant dose-dependent decrease in α1(I) collagen and TIMP-1 gene expression compared with activated HSCs treated with vehicle alone, while MMP-13 gene expression was increased (Figure 5). When expressed as a percentage of activated HSCs treated with vehicle alone (given the arbitrary value of 100%), one day of butein treatment at concentrations of 0.5 and 1.0 $\mu g\,mL^{-1}$ decreased α1(I) collagen to $80 \pm 10\%$ and $15 \pm 8\%$ ($P < 0.05$) and TIMP-1 to $30 \pm 8\%$ and $10 \pm 5\%$ ($P < 0.05$), respectively. When expressed as a percentage of activated HSCs treated with vehicle alone (given the arbitrary value of 1%), one day of butein treatment at concentrations of 0.5 and 1.0 $\mu g\,mL^{-1}$ increased MMP-13 to $4 \pm 0.6\%$ and $12 \pm 1\%$ ($P < 0.05$), respectively.

The degradation of extracellular matrix is initiated by MMPs. From the above results, the decrease in type-I collagen and α-SMA expression correlated with suppression of the mRNA expression levels of α1(I) collagen and TIMP-1, key molecules in hepatic fibrogenesis.

Discussion

Butein is a chalcone derivative. It has been reported that butein suppresses the proliferation of human colon adenocarcinoma and HeLa cell cultures (Ramanathan et al 1994; Yit et al 1994). Moreover, butein has an antioxidant effect (Sogawa et al 1994). These results led us to conduct experiments to find out whether butein could act directly on HSC activation and proliferation. HSC proliferation is a crucial step in the development of liver fibrosis. Proliferation of HSCs was induced either by oxidative stress (e.g. FeAsc) or by cytokine stimulation (e.g. PDGF-BB). HSCs were incubated with FeAsc, which induces the production of reactive-oxygen species. Several in-vitro and in-vivo studies have suggested that the formation of reactive-oxygen species could represent a common link between the different forms of chronic liver injury and hepatic fibrosis (Pietrangelo 1996; Svegliati-Baroni et al 2001). PDGF-BB, a potent proliferative cytokine (Pinzani et al 1989), was used to stimulate HSC proliferation. PDGF dimers are reported to play a pivotal role in mediating the proliferation of these cells. Accordingly, inhibitors that block PDGF- and FeAsc-stimulated HSC proliferation may provide potential new therapeutic strategies for the treatment of collagen deposition during chronic liver injury. The results presented here show that butein significantly inhibits the proliferation of activated HSCs.

Butein also inhibited the expression of α-SMA, the phenotypic marker of activated HSCs, in a dose-dependent manner. This shows that butein inhibits HSC activation. Additionally, butein reduced the production of collagen type-I protein. From this, it is evident that butein attenuates HSC activation and proliferation. We have also shown that butein has no toxic effects at the concentrations used in rat HSCs and hepatocytes (data not shown). Thus, activation of rat HSCs was found to be highly inhibited by butein, whereas rat hepatocytes were not sensitive to this compound.

We measured expression of α1(I)collagen, TIMP-1 and MMP-13 levels after butein treatment in activated HSC to evaluate the effect of butein on collagenolytic activity. The indicators of collagenolysis are several MMPs (MMP-1 in man, MMP-13 in rats) and their inhibitors (e.g. TIMP-1) (Milani et al 1994; Benyon et al 1996; Iredale et al 1996). In rat models, TIMP-1 is expressed early in fibrogenesis before apparent collagen deposition. In contrast to the TIMPs, mRNA for interstitial collagenase remains unaltered in human and rat liver as fibrogenesis develops. The resulting increase in the TIMP:MMP ratio in liver may promote fibrosis by protecting deposited extracellular matrix from degradation by MMPs.

Along this line, this study indicated that butein prevents liver fibrosis by suppressing the expression of TIMP-1 mRNA and by inducing the expression of MMP mRNA of HSC in fibrogenesis, resulting in reduced expression of collagen mRNA.

Conclusions

We evaluated the antifibrotic agent butein for its ability to block hepatic fibrosis in activated HSCs. A significant ameliorating effect of butein on hepatic fibrosis was demonstrated. Although the mechanism is not known, it is evident that butein significantly suppressed the induction of collagen and α-SMA expression at the protein level, and attenuated DNA synthesis in activated HSCs. The above results correlated with suppression of the mRNA expression levels of α1(I)collagen and TIMP-1, key molecules in hepatic fibrogenesis. On the basis of

these findings, we believe that butein may be a useful candidate for developing therapeutic agents for the prevention and treatment of collagen deposition in chronic liver injury.

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