ELSEVIER

Biochemical Pharmacology 63 (2002) 697–706

Biochemical Pharmacology

Inhibition of hepatic stellate cell collagen synthesis by *N*-(methylamino)isobutyric acid

Thomas L. Freeman[a,b,*], Kusum K. Kharbanda[a,b], Dean J. Tuma[a,b], Mark E. Mailliard[a,b]

[a]*Department of Internal Medicine, Section of Gastroenterology and Hepatology, University of Nebraska Medical Center, Omaha, NE 68105, USA*
[b]*Department of Veterans Affairs, VA Medical Center (151), Alcohol Research Center, 4101 Woolworth Ave., Omaha, NE 68105, USA*

Received 17 April 2001; accepted 2 July 2001

Abstract

The increased deposition of extracellular matrix by hepatic stellate cells following liver injury, in a process known as activation, is considered a key mechanism for increased collagen content of liver during the development of liver fibrosis. We report that *N*-(methylamino)isobutyric acid (MeAIB), a specific inhibitor of System A-mediated amino acid uptake, reduces the accumulation of collagen in CFSC-2G hepatic stellate cell cultures and in a rat model of liver injury and fibrosis. Rat CFSC-2G cells were cultured in 0–5 mM MeAIB, and the accumulation and synthesis of collagen were measured by binding to Sirius red F3B and pulse-labeling with [^{3}H]-proline, respectively. The effect of MeAIB on collagen accumulation *in vivo* was evaluated utilizing a rat model of hepatic fibrosis. MeAIB inhibited collagen accumulation in CFSC-2G cultures in a concentration-dependent manner with 5 mM MeAIB reducing collagen $44.6 \pm 1.2\%$ compared with the control. In CFSC-2G cultures, MeAIB selectively inhibited the incorporation of proline into cellular macromolecules by $43 \pm 4\%$, while the synthesis of proteins containing leucine was not affected. *In vivo*, oral administration of 160 mg MeAIB/kg body weight per day to rats significantly reduced the hepatic collagen accumulation in response to 1 week of CCl_4-induced liver injury. MeAIB reduces the accumulation of collagen in CFSC-2G hepatic stellate cell cultures and in a CCl_4-induced rat model of liver injury and fibrosis.

Keywords: Hepatic stellate cells; Liver fibrosis; Collagen; *N*-(Methylamino)isobutyric acid

1. Introduction

HSCs are central to the deposition of fibrillar and non-fibrillar ECM proteins following hepatocellular injury. Quiescent HSCs have a lipid-storing phenotype and express low levels of matrix proteins, but following hepatic injury HSC transform to a myofibroblast-like phenotype, proliferate, express α-smooth muscle actin (α-SMA), and produce large quantities of ECM proteins, notably procollagen-I, in a process termed activation. Because the excess production of matrix proteins by activated HSC is thought to be the most direct contribution of HSC to hepatic fibrosis, any method of attenuating matrix production would have a profound influence on the development of cirrhotic liver disease [1–3].

How activation of the HSC impacts the requirement of the cell for precursor molecules to fuel biosynthetic processes is largely unknown, but transformation from a quiescent lipid-storing cell to an activated myofibroblast, secreting large quantities of ECM protein, likely increases the demand of the cell for amino acids to fuel protein synthesis. In particular, the requirement for proline, which is highly enriched in collagen [4], is likely to be increased, and some investigations suggest that free proline is required for the efficient biosynthesis of collagen [5–7]. Proline enters the cell and is concentrated predominately via a Na^+-dependent amino acid transport system known as ''System A'' [8,9], and this transport system can be inhibited competitively with MeAIB, a non-metabolizable amino acid analog [9]. The effect of interfering with the uptake and subsequent intracellular concentration of amino acids on ECM protein synthesis by HSC is unknown, and the effect of inhibiting System A-mediated proline transport on collagen synthesis by HSC has not been studied.

* Corresponding author. Tel.: +1-402-346-8800x3735; fax: +1-402-449-0604.
E-mail address: tlfreeman@unmc.edu (T.L. Freeman).

Abbreviations: ECM, extracellular matrix; FBS, fetal bovine serum; HSCs, hepatic stellate cells; MeAIB, *N*-(methylamino)isobutyric acid; RLU, relative luciferase units; α-SMA, α-smooth muscle actin; TCA, trichloroacetic acid.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00885-1

Due to the importance of increased hepatic collagen in the development of cirrhosis, methods of inhibiting collagen synthesis could prove beneficial in the treatment of fibrotic liver disease. Proline analogs such as L-3,4-dehydroproline and L-azetidine-2-carboxylic acid have been reported to inhibit collagen synthesis [10] and to partially ameliorate hepatic cirrhosis induced in rats by CCl_4 [11], respectively. However, both of these compounds compete with proline during the aminoacylation of $tRNA^{pro}$ and are subsequently incorporated, in lieu of proline, into the growing peptide chains, a process that creates difficulties when separating the effects of amino acid uptake from those involving protein synthesis, modification, and secretion. MeAIB is a well-described inhibitor of proline uptake and is not metabolized or incorporated into cellular macromolecules [12], making it an ideal candidate for studying the influence of amino acid uptake and intracellular concentration on collagen biosynthesis.

We investigated the influence of MeAIB treatment on collagen production by a cell line derived from rat HSC, which displays a myofibroblast-like phenotype closely resembling activated HSC, as well as in an *in vivo* experimental model of hepatic fibrosis. Our findings indicate that MeAIB inhibits collagen synthesis by preferentially reducing the synthesis of proteins containing proline, and that oral administration of MeAIB to rats reduces the amount of collagen accumulated in the liver following CCl_4-mediated hepatic injury.

2. Materials and methods

2.1. Cell culture

The cell line designated CFSC-2G [13] was derived from rat liver, has a phenotype similar to that of early passage primary HSC, and was provided by M. Rojkind (Albert Einstein College of Medicine). CFSC-2G cells were routinely cultured in standard MEM formulated by combining MEM (Sigma, Cat. no. M0268) with 1× non-essential amino acids (Sigma, Cat. no. M-7145), 10 mM HEPES (pH 7.4), 10,000 unit penicillin with 10 mg streptomycin/L, and 10% FBS (Gibco/BRL). Ascorbate was added at 0.1 mM when indicated in the individual experiments. Incubation conditions were carried out at 37° with 5% CO_2 and 95% air. Cells were dissociated weekly in Ca^{2+}-free Hanks' basal salt solution containing 0.25% trypsin and 0.5 mM EDTA, and then plated onto plastic at a 1:4 dilution in standard MEM containing 10% FBS. The medium was changed biweekly.

2.2. Collagen assays

The collagen content of formalin-fixed samples was determined by the differential binding of Sirius red F3B and Fast green FCF to collagen and non-collagenous components, respectively, in the presence of picric acid [14,15]. Collagen content of paraffin-embedded rat liver sections was determined exactly as described by Jimenez *et al*. [14], and the collagen content of CFSC-2G HSC was determined by a modification of the original method. CFSC-2G HSCs were plated in 24-well dishes at a density that resulted in 70–80% confluence after a 24 hr incubation in standard MEM containing 10% FBS. Culture medium was then replaced with medium of the same type supplemented with 0.1 mM ascorbate containing supplemental amino acids at the concentration indicated in the individual experiments. Some cells were fixed at this time with 10% phosphate-buffered formalin to serve as a baseline when determining the change in collagen accumulation over the following test interval. After 24 hr, the medium was aspirated, and the cells were fixed in 10% phosphate-buffered formalin. Cells were stained at room temperature as follows: (i) 15 min in 0.5 mL of a solution containing 0.01% Fast green FCF, 0.5% picric acid in distilled water; (ii) 15 min in 0.5 mL of a solution containing 0.04% Fast green FCF, 0.1% Sirius red, 0.5% picric acid in distilled water; and (iii) washing in tap water for 5–10 min until the elution fluid was completely clear. Cells were allowed to dry before elution of the bound dyes with 1 mL of 50% methanol containing 50 mM NaOH. After a spectrum scan to establish the peak absorbances for each dye, the absorbance of the solution at 630 and 540 nm was determined by a spectrophotometer. Calculation of collagen and protein content was performed exactly as described [14], and the data are expressed as the change in collagen content/mg total protein over the treatment interval to correct for collagen present in the cultures before the addition of the test compounds.

2.3. Measurement of collagen synthesis rate

The rate of collagen synthesis in CFSC-2G HSC was determined by measuring the amount of L-[2,3,4,5-^{3}H]-proline (American Radiolabeled Chemicals, Inc.) incorporated into collagenase digestible macromolecules [16,17]. Cells were cultured in 24-well dishes and exposed to 0.25 mL MEM (contains 0.1 mM non-labeled proline) supplemented with 0.1 mM ascorbate, 5 μCi [^{3}H]-proline, and amino acids as described in the individual experiments. After 2 hr of labeling, 0.25 mL of stop buffer (0.2% SDS containing 0.2 M NaOH) was added to terminate the labeling reaction and to solublize the cells. The lysate, which included secreted and intracellular collagen, was incubated at 80° for 10 min to inactivate protease activity and was then passed over a Sephadex G50 spin column equilibrated with TBS (100 mM NaCl, 10 mM Tris, pH 7.5) to remove the unincorporated [^{3}H]-proline and to change to a buffer suitable for digestion with purified collagenase. Collagen in the sample was digested for 4 hr at 37° by combining 3.5×10^4 to 5×10^4 dpm labeled macromolecules with 5 unit of purified collagenase (form III, Advance Biofactures Corp.) and supplementing to

0.1 mM with $CaCl_2$. Collagenase-resistant material was precipitated with 10% trichloroacetic acid (TCA), and the soluble fraction was counted to quantify the [^{3}H]-proline liberated by collagenase digestion. Mock incubations were done in parallel to correct for background, and data were normalized to [^{3}H]-proline incorporated into TCA-insoluble material prior to collagenase digestion.

2.4. α2(I) procollagen reporter gene assay

The expression of the *α2(I)* procollagen gene was monitored by transiently transfecting CFSC-2G HSC with a reporter plasmid, pGL1009, provided by Benoit de Crombrugghe (Anderson Hospital and Tumor Institute) as modified by Estaban Mezey and James Potter (Johns Hopkins University School of Medicine). Cells were plated on 24-well dishes to produce a density of 10–20% after a 24 hr incubation in MEM containing 10% FBS. Transfection of CFSC-2G cells with pGL1009 [18] utilized the SuperFect™ reagent (QIAgen). The medium was aspirated and replaced with 0.25 mL of serum-free MEM containing 1 μg plasmid DNA and 4 μL SuperFect™ reagent/mL. After incubation at 37° for 60 min, 1 mL MEM containing 10% FBS was added to each well, and the cells were incubated for an additional 24 hr, which resulted in a density of 70–80% confluence. Medium was aspirated and replaced with identical medium containing 0 or 5 mM MeAIB, and luciferase activity was measured after a 12 hr incubation. Luciferase activity was assayed according to the instructions of the manufacturer using the Luciferase Assay System (Promega) and a TD-20/20 Luminometer (Turner Designs).

2.5. Zymography of collagenase activity

Collagen-degrading activity in culture medium conditioned by CFSC-2G HSC was assessed by zymography. Cells were plated in 24-well dishes with MEM and 10% FBS to yield 70–80% confluent cultures after 24 hr. The medium was aspirated and replaced with 0.3 mL of MEM supplemented with 0.1 mM ascorbate, 10% FBS, and amino acids as described in the individual experiments. Some experiments used serum-free conditions at this point when indicated. Cells were incubated at 37° with 5% CO_2 and increased humidity to minimize evaporation of the medium. The medium was collected after 24 hr and frozen at −70°. Zymography of secreted collagen degrading enzymes was conducted as described [19,20]. In brief, secreted proteins were resolved by non-reducing, native SDS-PAGE through an 8% resolving gel containing 0.1% gelatin (Sigma, porcine skin, type A, bloom 300), renatured at room temperature by incubating the gel for 30 min in several volumes of 2.5% Triton X-100, and developed by incubation at 37° for 16–18 hr in development solution (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 0.06% Brij-35, pH 8). Bands of enzyme activity were visualized by staining the gel with Coomassie blue (40% methanol, 10% acetic acid with 0.5% Coomassie brilliant blue R-250) and de-staining with 40% methanol containing 10% acetic acid.

2.6. Determination of steady-state MeAIB, proline, and leucine levels

The change in the steady-state level of intracellular amino acids was measured by a modification of a whole cell transport assay [21]. Cells were plated in 24-well dishes at a density to result in 70–80% confluence following incubation for 18–24 hr in MEM containing 10% FBS. The medium was aspirated and replaced with identical medium supplemented with a mixture of labeled and unlabeled test amino acid to produce a concentration of 0.2 mM and a specific activity of 1 μCi/mL (∼5 mCi/mmol final), and the unlabeled competing amino acid was added at 0, 0.1, 0.5, 1, or 5 mM. The cells were incubated at 37° with 5% CO_2 for 90 min to ensure that steady-state conditions were achieved. Extracellular label was removed with three rapid washes in 1 mL of ice-cold Krebs–Ringer bicarbonate buffer (Sigma). Cells were disrupted with 0.2 mL of 0.1% SDS/0.1 M NaOH, and the released labeled amino acid was quantified by scintillation counting. Protein content was determined by the Lowry method for normalization, and the incorporation of label into macromolecules was corrected by measuring the TCA-insoluble radioactivity. [*N-Methyl*-^{3}H]aminoisobutyric acid (60–80 mCi/mmol), L-[2,3,4,5-^{3}H]-proline (90–120 Ci/mmol), and L-[4,5-^{3}H(*N*)]-leucine were purchased from American Radiolabeled Chemicals, Inc., and unlabeled amino acids of the highest available purity were purchased from the Sigma.

2.7. Measurement of proline and leucine incorporation into protein

The rate at which proteins containing leucine or proline were synthesized was measured by pulse-label experiments. CFSC-2G HSCs were exposed to standard MEM containing 10% FBS, 20 μCi/mL of [^{3}H]-proline or [^{3}H]-leucine, and either 0 or 5 mM MeAIB for 60 min. The medium was aspirated, and the cells were solublized in 0.1% SDS containing 0.1 M NaOH. Cell lysates were combined with 100 μg of carrier protein (BSA) and precipitated with 10% TCA. Precipitated proteins were collected by filtration under vacuum over glass fiber disks followed by two washes in ice-cold 5% TCA and two washes with acetone. Radioactivity in macromolecules was measured by scintillation counting.

2.8. In vivo model of liver fibrosis

Our experimental model of CCl_4-induced liver fibrosis was adapted from the methods described by Iredale

et al. [22]. Adult male Sprague–Dawley rats (200–250 g) were obtained from Harlan Sprague Dawley. Protocols were approved by the Omaha VA Medical Center Institutional Animal Care and Use Committee and complied with the guidelines set by the National Institutes of Health. Rats were maintained under a controlled temperature with a 12 hr light–dark cycle for at least 5 days before use in experiments. Standard rodent chow and de-ionized water were provided *ad libitum*. Induction of liver injury and progressive fibrosis were produced by biweekly intraperitoneal injections of 0.2 mL CCl_4, in a 1:1 (v/v) ratio with peanut oil. MeAIB was administered by inclusion of 1 g MeAIB/L of drinking water for 3 days prior to the first CCl_4 injection. Animals were anesthetized with 100 mg ketamine containing 0.1 mg acepromazine, and tissues were harvested 10 and 17 days following the first CCl_4 injection. Injections of CCl_4 were scheduled so that livers were collected 3 days following the last CCl_4 treatment. Control livers were harvested from rats 3 days following MeAIB treatment and were not exposed to CCl_4. Blood was collected by heart puncture and stored at -70° following removal of the non-serum components by centrifugation. One lobe of each liver was fixed in buffered formalin for production of paraffin-embedded sections, while the remainder was stored frozen at -70° until needed for immunoblotting.

2.9. Western blotting

α-SMA was detected in liver homogenates by standard Western blotting [23], utilizing a monoclonal anti-α-SMA antibody probe (clone 1A4, Sigma). Liver samples were homogenized by grinding in a buffer containing 1% SDS, 0.1 mM EDTA, and 10 mM Tris (pH 8) followed by incubation at 80° for 20 min. Insoluble material was removed by centrifugation (10 min at 10,000 *g* at room temperature), and protein content was determined by the Lowry assay. Liver proteins (100 μg) were separated by denaturing SDS-PAGE and transferred to nitrocellulose by electro-blotting. Nitrocellulose was blocked with 2% BSA in TBST (150 mM NaCl, 10 mM Tris, 0.02% Tween 20, pH 7.5) for 2 hr followed by incubation with primary anti-α-SMA at 4° for 16–18 hr. Visualization of primary antibody was with alkaline phosphatase-conjugated anti-mouse IgG and BCIP/NTB using standard methods.

2.10. Statistical analysis

Each data point represents the mean ± SD of a minimum of three determinations or the number of replicates (*N* value) indicated in the individual experiments. Significance was evaluated utilizing Student's *t*-test with $P < 0.01$ and $P < 0.05$ set as statistically significant *in vitro* and *in vivo*, respectively.

3. Results

3.1. Collagen accumulation in cultured CFSC-2G cells

Differential binding of Sirius red F3B and Fast green FCF to collagen and non-collagenous components was utilized to quantify the amount of collagen present in CFSC-2G cultures [14,15]. These results were confirmed by continuous labeling with [^{3}H]-proline over 24 hr followed by determination of the collagen content [16,24], which produced representative results (data not shown). As illustrated in Fig. 1, the addition of MeAIB reduced the amount of collagen present in cultures of CFSC-2G HSC. The effect was concentration-dependent with concentrations of MeAIB 1 mM or below having little or no effect on the accumulation of collagen by the cells over a 24 hr period. MeAIB concentrations at or above 2.5 mM decreased the amount of collagen by 40.5 ± 1.8, 44.6 ± 1.2, and $42.1 \pm 2.3\%$ compared with control at 2.5, 5, and 10 mM MeAIB, respectively. The effect of MeAIB on the accumulation of collagen by CFSC-2G HSC was maximal at 5 mM with higher concentrations of MeAIB unable to inhibit collagen production further. MeAIB did not reduce significantly the total amount of protein measured by Fast green FCF binding in samples treated with 5 mM or less MeAIB, but 10 mM MeAIB reduced the amount of total protein by $6.8 \pm 1.7\%$. Since 5 mM MeAIB was the lowest concentration that resulted in a maximal reduction in collagen accumulation over a 24 hr period without interfering with non-collagenous protein synthesis, this concentration was used in all subsequent experiments.

3.2. Inhibition of collagen synthesis by MeAIB

Because 5 mM MeAIB reduced the accumulation of collagen in CFSC-2G HSC, the effect of MeAIB on the

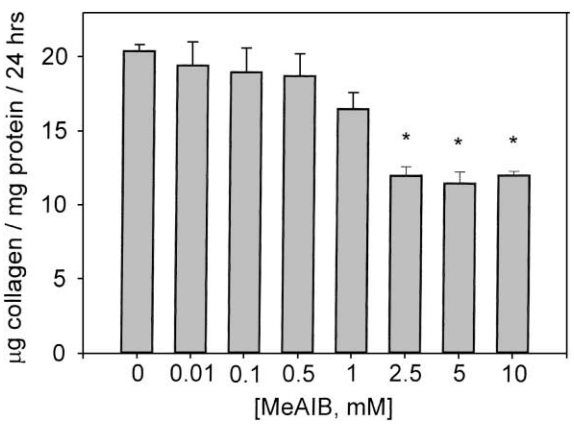

Fig. 1. Collagen accumulation by CFSC-2G cultures. CFSC-2G cells were cultured in medium supplemented with the indicated concentration of MeAIB as described in Section 2. Asterisks indicate significance by Student's *t*-test at $P < 0.01$ vs. 0 mM MeAIB. Data represent the means ± SD of three independent experiments ($N = 9$).

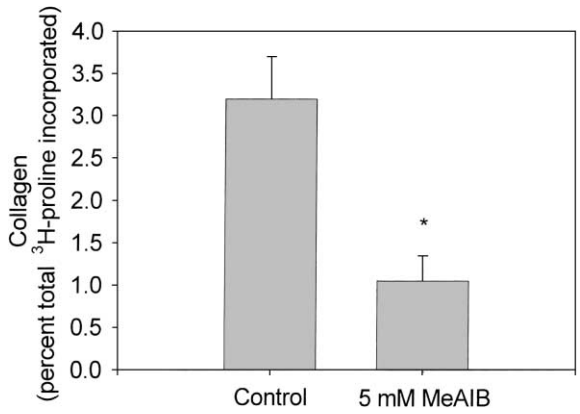

Fig. 2. Collagen synthesis rate by cultures of CFSC-2G cells. Collagen synthesis was measured with and without 5 mM MeAIB by incorporation of [^{3}H]-proline into collagenase-digestible macromolecules as described in Section 2. Data are normalized to total incorporation of [^{3}H]-proline into TCA-insoluble material before digestion. The asterisk indicates significance by Student's *t*-test at $P < 0.01$. Data are the means ± SD of three determinations and are representative of three independent experiments.

rate of collagen synthesis was investigated as a possible cause for this reduction. The sensitivity of proteins pulse-labeled with [^{3}H]-proline to collagenase was utilized as a measure of the collagen synthesis rate of CFSC-2G cells. As shown in Fig. 2, 3.2 ± 0.5% of the proline incorporated into protein over a 2 hr period was directed to collagen. MeAIB (5 mM) reduced the synthesis of collagen to 1.1 ± 0.4% of the cellular proline incorporation. These results indicate that inclusion of 5 mM MeAIB decreased the incorporation of [^{3}H]-proline into collagen by 2.9-fold, demonstrating a profound inhibitory effect of MeAIB on collagen synthesis by CFSC-2G cells.

3.3. α2(I) procollagen gene expression

The effect of the amino acid analog MeAIB on collagen synthesis could potentially result from perturbation of transcriptional or post-transcriptional processes. The expression of the *α2(I)* procollagen gene was investigated to examine changes in collagen gene expression following MeAIB treatment. A reporter gene, luciferase, fused to the *α2(I)* procollagen promoter was used to quantify any changes in gene expression. The addition of 5 mM MeAIB did not have an effect on gene expression under the control of the *α2(I)* procollagen promoter. Transfected CFSC-2G cells expressed 4.4 ± 0.78 RLU/mg protein after 12 hr in control medium, and paired cultures expressed 4.8 ± 0.92 RLU/mg protein after 12 hr in medium containing 5 mM MeAIB. These data show that decreased collagen synthesis in CFSC-2G cells following MeAIB treatment could not be attributed to an attenuation in the transcriptional expression by the *α2(I)* procollagen promoter.

3.4. Secretion of collagen-degrading enzymes

The net accumulation of collagen is dependent upon the rate of collagen synthesis and the rate of collagen degradation. Since the accumulation and synthesis of collagen were inhibited by MeAIB, experiments were conducted to examine any change in the collagen-degrading activity in culture medium harvested from CFSC-2G cells. Zymography was utilized to: (i) measure the amount of collagen-degrading activity in culture medium conditioned by CFSC-2G cells; and (ii) provide additional information on the different species of collagen-degrading enzymes produced. An unexpected finding was the large amount of collagenase activity present in unconditioned medium containing 10% FBS (lane 7, Fig. 3A). In medium containing 10% FBS, the preponderance of collagen-degrading activity was derived from the bovine sera and not the CFSC-2G HSC. However, the collagen-degrading activity attributable to the CFSC-2G cells did not change with the inclusion of 5 mM MeAIB or 5 mM alanine plus 5 mM MeAIB as seen in Fig. 3A by comparing the activity found in conditioned medium (lanes 1–6) with the activity found in unconditioned medium (lane 7). To better visualize the collagenase activity in medium conditioned by CFSC-2G HSC, the experiment was duplicated under serum-free

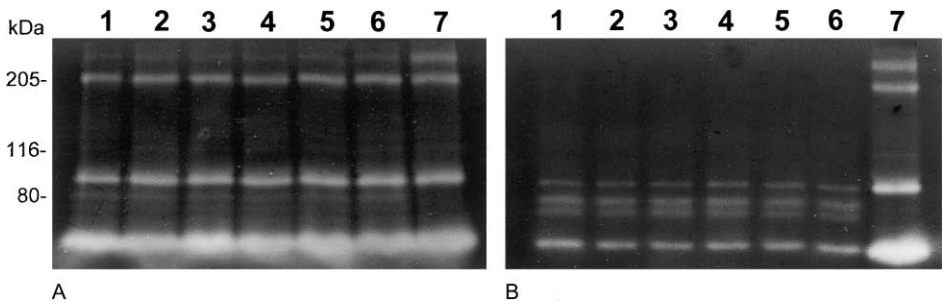

Fig. 3. Collagen-degrading activity of CFSC-2G cells. Collagenase activity in culture medium conditioned by CFSC-2G cells was assayed by zymography as described in Section 2. Medium containing 10% FBS (panel A) or serum-free medium (panel B) were conditioned by incubation with CFSC-2G cell cultures for 24 hr before determination of secreted collagenase activity by zymography. Treatment conditions were control medium (lanes 1 and 2), medium containing 5 mM MeAIB (lanes 3 and 4), or medium containing both 5 mM MeAIB and 5 mM alanine (lanes 5 and 6). Unconditioned medium containing 10% FBS was included in lane 7 as a reference. Cells were plated in a paired manner, and treatments were performed in duplicate. Results are representative of two experiments.

conditions as described in Section 2. MeAIB did not change the collagen-degrading activity in serum-free CFSC-2G cell-conditioned medium, as seen in Fig. 3B. Because the collagen-degrading activity of CFSC-2G HSC conditioned medium did not change in response to treatment of the cells with 5 mM MeAIB, the reduction in collagen accumulation resulting from MeAIB treatment could not be attributed to altered production of collagen-degrading enzymes.

3.5. Effect of exogenous amino acids on collagen

Since MeAIB inhibits the uptake of small neutral amino acids into the cell by *cis* and *trans* effects on the System A transporter [9], we reasoned that providing excess amounts of exogenous amino acids may reverse the effect of MeAIB on collagen synthesis. However, alanine, glycine, and proline were unable to reverse the effect of MeAIB on the accumulation of collagen in cultures of CFSC-2G HSC. As seen in Fig. 4, the addition of 5 mM alanine, proline, or glycine did not reverse the 40–50% reduction in collagen accumulation in CFSC-2G HSC cultures. Moreover, the simultaneous addition of 5 mM alanine, proline, and glycine had no significant effect on collagen accumulation in the absence of MeAIB. Additional experiments found that the simultaneous addition of 5–10 mM alanine, proline, and glycine was no more effective at reversing the effect of MeAIB than when single amino acids were used. These data indicate that the effect of MeAIB on collagen accumulation is not reversible by the addition of exogenous amino acids, and also that exogenous alanine, proline, and glycine do not inhibit collagen accumulation in CFSC-2G HSC cultures in the absence of MeAIB.

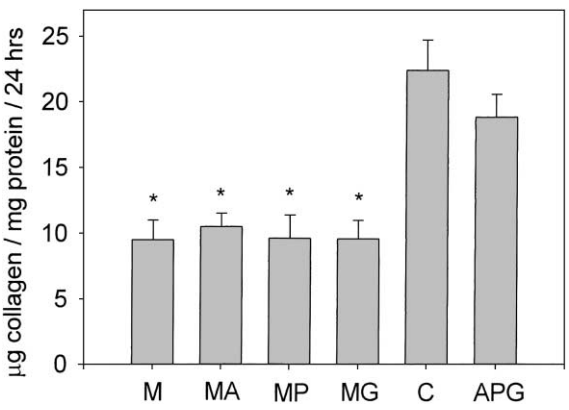

Fig. 4. Effect of exogenous amino acids on collagen accumulation. CFCS-2G HSCs were incubated for 18–24 hr in medium containing 5 mM MeAIB (M), 5 mM MeAIB plus 5 mM alanine (MA), 5 mM MeAIB plus 5 mM proline (MP), 5 mM MeAIB plus 5 mM glycine (MG), no supplemental amino acids (C), or 5 mM alanine, 5 mM proline, plus 5 mM glycine (APG). Collagen content was determined by the differential binding of Sirius red F3B and Fast green FCF to collagen and non-collagenous components, respectively, in the presence of picric acid as described in Section 2. Data are the means ± SD of four replicates and are representative of three independent experiments. Asterisks indicate significance at $P < 0.01$ vs. no supplemental amino acids (C).

3.6. Intracellular MeAIB, proline, and leucine levels

Because exogenous amino acids were not able to reverse, or even partially reverse, the inhibitory effect of MeAIB on collagen accumulation, studies were conducted to examine the influence of MeAIB on the intracellular level of naturally occurring amino acids at steady-state conditions. The steady-state accumulations of proline and leucine were chosen for study because MeAIB is known to inhibit the uptake of proline competitively, while having little or no inhibitory effect on leucine uptake [9]. As expected, MeAIB reduced the accumulation of intracellular proline in a concentration-dependent manner. MeAIB had virtually no effect on the accumulation of intracellular leucine (Fig. 5A). In the absence of MeAIB, proline accumulated to a high level of 5.28 ± 0.37 nmol/mg protein due to the active transport of proline in a process dependent upon the Na^+ gradient of the plasma membrane. Addition of 0.1–5 mM MeAIB reduced the intracellular proline level by a maximum of 54 ± 7%. The steady-state level of leucine was lower than that of proline due to the passive uptake of leucine by the Na^+-independent leucine transport system, and the addition of MeAIB did not alter

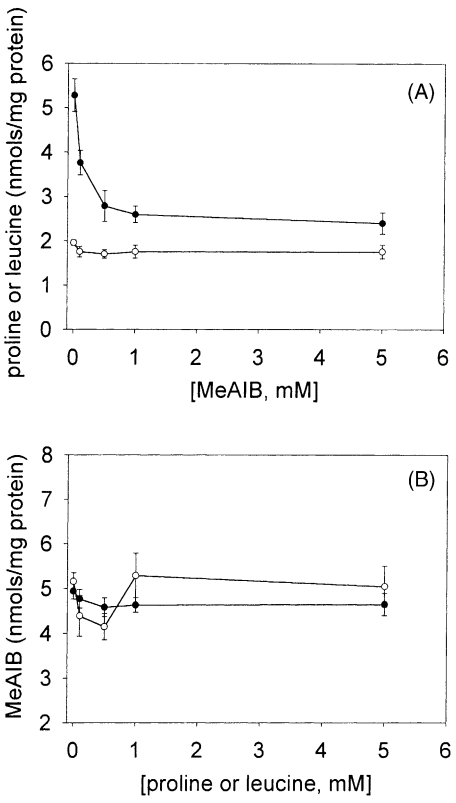

Fig. 5. Intracellular proline and leucine content of CFSC-2G cells. Intracellular proline, leucine, and MeAIB were measured utilizing radioactive tracers as described in Section 2. Panel A illustrates the effect of 0–5 mM MeAIB on intracellular proline (●) or leucine (○) levels. Panel B shows the effect of 0–5 mM proline (●) or 0–5 mM leucine (○) on intracellular MeAIB accumulation. Data are the means ± SD of two independent experiments composed of four replicates ($N = 8$).

the intracellular level of leucine significantly. Experiments were also done to examine the ability of proline or leucine to influence the accumulation of intracellular MeAIB. Paradoxically, proline was not able to decrease the intracellular level of MeAIB (Fig. 5B). MeAIB accumulated to 4.94 ± 0.18 nmol/mg protein, a level nearly identical to that reached by proline. Leucine, as expected, did not have an effect on the intracellular level of MeAIB. These findings demonstrate that MeAIB inhibits the intracellular accumulation of proline, but proline is unable to influence the accumulation of intracellular MeAIB.

3.7. Proline and leucine incorporation into protein

Because MeAIB reduced the intracellular level of proline while leaving the level of leucine unchanged, the hypothesis that synthesis of proteins containing proline should be affected to a greater degree by MeAIB than proteins containing little or no proline was investigated. This idea was tested by pulse-labeling CFSC-2G HSC with [^{3}H]-proline or [^{3}H]-leucine in the presence or absence of 5 mM MeAIB. As summarized in Fig. 6, the incorporation of [^{3}H]-proline into protein was inhibited $43 \pm 4\%$ by 5 mM MeAIB, but MeAIB had no effect on the incorporation of [^{3}H]-leucine into TCA-insoluble macromolecules. This result demonstrates that proteins containing proline are more sensitive to the effect of MeAIB than proteins containing little or no proline.

3.8. Collagen accumulation in vivo

Studies were also conducted to determine if the MeAIB-mediated inhibition of collagen synthesis and accumulation in CFSC-2G HSC produced similar results in an animal model of hepatic injury and fibrosis. While measuring collagen synthesis rates by HSC *in vivo* presents a significant technical challenge, the measurement of collagen accumulation in liver sections following injury is relatively straightforward; therefore, the amount of collagen/mg of liver protein was used as a measure of effectiveness in the *in vivo* model. Addition of 1 g MeAIB/L of drinking water reduced the hepatic collagen content of liver in a CCl_4-induced model of hepatic fibrosis (Fig. 7). Both control and MeAIB-treated animals had increased hepatic collagen following 1 and 2 weeks of CCl_4 administration compared with liver before treatment. Control animals contained 131 ± 18 and 155 ± 12 μg collagen/mg liver protein, while MeAIB treatment reduced the hepatic collagen to 108 ± 18 and 128 ± 15 μg collagen/mg liver protein following 1 or 2 weeks of liver damage, respectively. While the reduction in hepatic collagen was relatively modest, MeAIB did produce a statistically significant reduction 1 week into the *in vivo* model of liver fibrosis. Despite an apparent reduction in collagen, the amount of hepatic collagen present 2 weeks into the treatment just missed statistical significance between the two groups with $P < 0.06$.

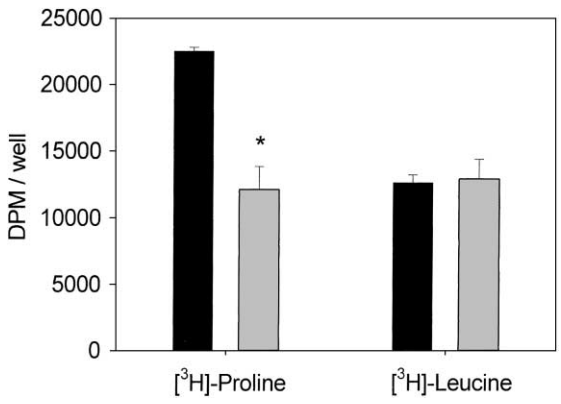

Fig. 6. Incorporation of proline or leucine into macromolecules. The incorporation of proline or leucine into CFSC-2G cellular proteins was measured by pulse-label experiments as described in Section 2. Control medium and medium containing 5 mM MeAIB are indicated by black or gray bars, respectively. Data are the means ± SD of four replicates and are representative of three independent experiments. The asterisk indicates significance at $P < 0.01$.

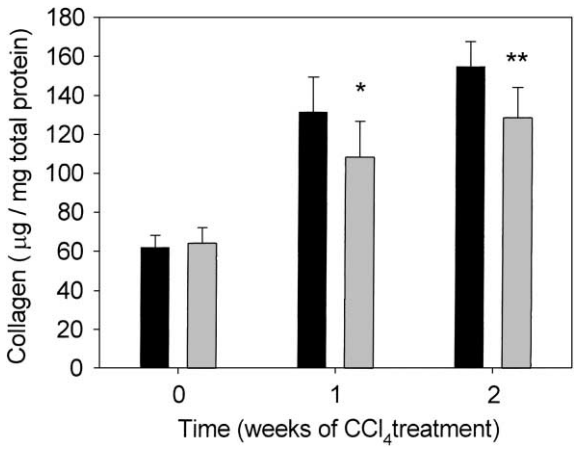

Fig. 7. Collagen content in the liver of CCl_4-treated rats. Animals were given drinking water (black bars) or 1 g MeAIB/L of drinking water (gray bars) for 3 days before treatment with CCl_4, and the liver collagen content was determined 0 (before liver damage), 1, and 2 weeks following experimental liver damage as described in Section 2. Data are the means ± SD of four determinations. P values are indicated by asterisks: $P < 0.04$ (*) and $P < 0.06$ (**).

3.9. α-SMA levels following liver injury

An indication of HSC activation is the *de novo* synthesis of α-SMA [2]. Western blotting revealed that α-SMA was increased following CCl_4-induced liver injury, and that oral administration of MeAIB reduced the α-SMA present in liver, indicating that MeAIB potentially inhibited the activation of HSC (Fig. 8).

3.10. Blood liver enzymes following liver injury

Blood GOT enzyme activity was monitored as an indicator of hepatocyte injury in response to the CCl_4

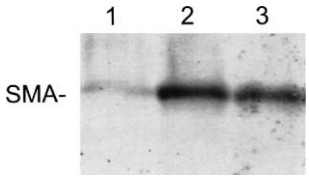

Fig. 8. α-SMA in liver following CCl_4-induced hepatic injury. Animals were treated as described in the legend of Fig. 7, and livers were harvested after 1 week of CCl_4 treatment. α-SMA was detected in homogenized liver by Western blotting. Lane 1 contains liver proteins from animals before CCl_4 treatment as a reference. Lanes 2 and 3 contain liver samples from animals receiving drinking water or 1 g MeAIB/L of drinking water, respectively, beginning 3 days before CCl_4 treatment. Data are representative of three experiments.

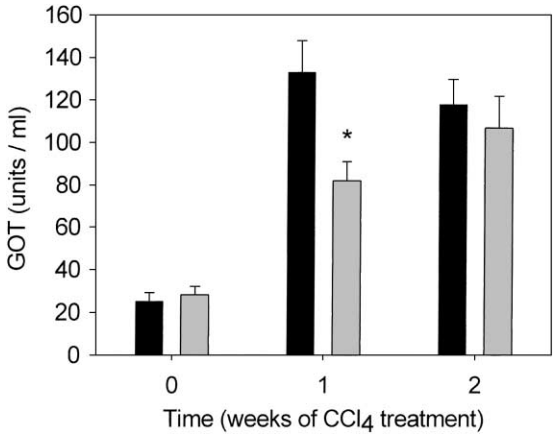

Fig. 9. GOT enzyme activity in blood of CCl_4-treated rats. Animals were provided drinking water (black bars) or 1 g MeAIB/L of drinking water (gray bars) exactly as described in the legend of Fig. 7, and blood plasma levels of the liver enzymes were determined. Data are the means ± SD of four determinations. The asterisk indicates significance at $P < 0.01$.

injections. We found that oral treatment with MeAIB reduced the GOT enzyme level present in the serum fraction of blood by $38 \pm 11\%$ after 1 week of CCl_4-induced liver injury (Fig. 9). The hepato-protective effect of MeAIB was not observed after 2 weeks of CCl_4-induced liver injury.

4. Discussion

We report here that MeAIB treatment effectively reduces the accumulation of collagen in an *in vitro* model of activated HSC and in an *in vivo* rat model of liver injury and fibrosis. MeAIB was found to decrease collagen accumulation in cultures of CFSC-2G HSC by inhibiting the synthesis of cellular proteins containing proline. Attenuated gene expression of *α2(I)* procollagen or increased secretion of collagen-degrading enzymes could not account for the reduction in collagen accumulation in response to MeAIB treatment. *In vivo*, oral administration of ~160 mg MeAIB/kg per day to rats significantly reduced the hepatic collagen accumulation resulting from 1 week of CCl_4-mediated liver injury. MeAIB also displayed a hepato-protective property as demonstrated by reduced GOT enzyme activity found in peripheral blood following 1 week of liver injury. The decrease in α-SMA expression in liver following CCl_4-mediated injury resulting from MeAIB treatment indicated that MeAIB also potentially interfered with HSC activation either directly or indirectly by reducing hepatic damage. These data provide evidence that mechanisms of reducing the availability of proline to HSC can provide a novel method for inhibiting collagen synthesis and accumulation in response to hepatic injury.

The inhibition of collagen accumulation by MeAIB appears to result from selectively impairing the synthesis of proteins containing proline residues. The results of this study showed that the incorporation of proline into CFSC-2G cell proteins was inhibited nearly 50% by 5 mM MeAIB while the incorporation of leucine was unaffected. Neither *α2(I)* procollagen promoter-driven gene expression nor collagen-degrading enzyme activity was affected by including 5 mM MeAIB in the culture medium. Because MeAIB did not appear to affect general protein synthesis, as seen by the lack of an effect by MeAIB on the incorporation of leucine into proteins, and because many proteins other than collagen contain proline residues, it is reasonable to speculate that proteins containing large numbers of proline residues should be more sensitive to the inhibitory effect of MeAIB than proteins containing very few prolines. Our finding, that MeAIB can reduce the accumulation and synthesis of collagen (a protein enriched with proline residues) while having no measurable effect on non-collagenous proteins, supports this idea.

Because MeAIB and proline share the same transport system to gain entry into the cell [8,9], a plausible explanation for the reduction in proline incorporation into protein is the limitation of proline availability for protein synthesis due to the effect of MeAIB on the uptake and subsequent cytoplasmic accumulation of proline. MeAIB reduced the intracellular concentration of proline, in a concentration-dependent manner, by a maximum of 55% in CFSC-2G cells, while the intracellular accumulation of leucine was not affected by MeAIB. These data correlate well with the incorporation of proline and leucine into proteins in the presence of 5 mM MeAIB.

Care must be taken not to assign all of the effect exerted by MeAIB solely to inhibiting the uptake of proline, because MeAIB has been described previously to have the unusual property of displacing intracellular amino acids while not being influenced by the presence of other amino acids [9]. In this regard, MeAIB effectively decreased the intracellular concentration of proline, but proline was not able to reduce the intracellular concentration of MeAIB. The inability of exogenous alanine, proline, or glycine to even partially reverse the effect of MeAIB on collagen accumulation in CFSC-2G cultures provides additional evidence indicating that simple

competitive inhibition of amino acid uptake by MeAIB may not fully explain the inhibitory properties of MeAIB on the synthesis of collagen.

While the mechanism of this ability of MeAIB to reduce intracellular amino acid accumulation without being displaced itself is not fully understood, this property likely results in a further reduction in the cellular availability of specific amino acids and influences the physiology of the cell in ways that are difficult to predict. We hypothesize that MeAIB treatment results in an insufficient supply of the amino acids that predominantly gain entry into the cell via the System A amino acid uptake pathway, of which proline is the prime example. This would lead to a decreased number of aminoacylated $tRNA^{pro}$ molecules eventually impacting the synthesis of proteins containing proline. It is unlikely that the mechanism involves an alteration in the behavior of ribosomes, or a generalized down-regulation in protein synthetic rates, because the incorporation of leucine into protein was unaffected by MeAIB, indicating a process selective for proteins containing proline. Despite incomplete information on the precise mechanism for selectively inhibiting the synthesis of proline-containing proteins, MeAIB did inhibit effectively the production of proline-enriched proteins, such as collagen, in CFSC-2G cells as well as in liver following experimental injury.

In the rat model of hepatic injury and fibrosis, oral administration of MeAIB also resulted in the decreased accumulation of liver collagen. Significant reduction in hepatic collagen was observed after 1 week of CCl_4-induced liver injury, but MeAIB treatment did not produce a statistically significant change in hepatic collagen content after 2 weeks of treatment. While the 18% decrease in total hepatic collagen due to MeAIB appears modest after 1 week of treatment, the inhibition of the collagen increase (i.e. when compared with liver collagen content before injury) was more telling with collagen increasing 211 ± 32 and $168 \pm 21\%$ in control and MeAIB-treated animals, respectively. This indicates that MeAIB inhibited the increase in hepatic collagen, above the uninjured liver content, by as much as 38–43% over the first week of treatment. Why MeAIB treatment did not produce statistically significant reductions in hepatic collagen 2 weeks into treatment is unclear, but the data did maintain a downward trend and only missed significance slightly ($P < 0.06$). How modification of the dosage of MeAIB would influence the accumulation of collagen was not studied, but it is likely that increasing the daily dose of MeAIB would reduce the accumulation of hepatic collagen further, possibly resulting in statistically significant reductions beyond the first week of treatment.

Previous studies report that much higher doses of MeAIB produce no obvious toxic side-effects in rats [12,25]. In this study, including 1 g MeAIB/L of drinking water produced a daily dose of 159 ± 33 mg MeAIB/kg body weight as calculated from the daily water utilization. Water intake was not significantly different between the groups (data not shown). This dose is quite modest compared with reports in the literature giving MeAIB to rats at doses of 1.17 g/kg per day for 8 consecutive days [12]. Also, rats do not have increased mortality rates following resection of 68–70% of their liver with the concurrent intraperitoneal injection of up to 10 g MeAIB/kg [25], which is 60 times greater than the oral dose given in this study. Because higher doses of MeAIB can be well tolerated by the rat, even under conditions of reduced liver capacity, it is quite likely that increasing the dose of MeAIB could further reduce the hepatic collagen content without exhibiting an overt toxic effect. However, this has yet to be tested experimentally and was beyond the scope of this study.

In addition to reducing the hepatic collagen content, the decreased expression of α-SMA by MeAIB treatment in liver 1 week following CCl_4-induced liver injury indicates that this agent potentially interfered with the activation of HSC. Furthermore, previous studies by our laboratory indicate that MeAIB can inhibit the proliferation of hepatocytes following partial hepatectomy [25]. Because HSC activation is closely coupled to HSC proliferation [2], MeAIB treatment could have the additional property of inhibiting HSC activation, provided that the antiproliferative properties of MeAIB extend to HSC. In fact, unpublished experiments by our laboratory indicate that MeAIB treatment partially inhibits the rapid induction of a proliferative burst by serum-starved CFSC-2G cultures following stimulation with 10% FBS.

It is interesting to also note that the MeAIB-mediated reduction in hepatic α-SMA following CCl_4-induced liver injury could indicate reduced activation of quiescent HSC due to the apparent hepato-protective property of MeAIB. Because MeAIB treatment reduced the blood level of enzymes indicative of hepatic injury, the degree of hepatic injury was likely smaller in the MeAIB-treated animals, which would suggest attenuated liver damage and corresponding hepatic inflammation, the key inducer of HSC activation [26,27]. How MeAIB produces a hepato-protective effect is not known, but similar hepato-protective observations have been made for molecules with similar structures, such as betaine [28], alanine [29], and glycine [30]. Overall, these data indicate that MeAIB may reduce hepatic collagen accumulation by preventing HSC activation, either directly or indirectly by preventing liver injury, in addition to inhibiting collagen production by activated HSC.

References

[1] Gressner AM. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis. Kidney Int 1996;49:S39–45.

[2] Li D, Friedman SL. Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. J Gastroenterol Hepatol 1999;14:618–33.

[3] Eng FJ, Friedman SL, Fibrogenesis I. New insights into hepatic stellate cell activation: the simple becomes complex. Am J Physiol Gastrointest Liver Physiol 2000;279:G7–G11.
[4] Prockop DJ, Kivirakko KI, Tuderman L, Guzman N. The biosynthesis of collagen and its disorders. New Engl J Med 1979;301:13–23.
[5] Popper H, Udenfriend S. Hepatic fibrosis correlation of biochemical and morphologic investigation. Am J Med 1970;49:707–21.
[6] Rojkind M, Diaz de León L. Collagen biosynthesis in cirrhotic rat liver slices: a regulatory mechanism. Biochim Biophys Acta 1970;217:512–22.
[7] Blumenkrantz N, Asboe-Hansen G. Effect of amino acids on collagen biosynthesis. In Vitro 1973;8:342–6.
[8] Oxender DL, Christensen HN. Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. J Biol Chem 1963;238:3686–99.
[9] Christensen HN. Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Rev 1990;70:43–77.
[10] Kerwar SS, Felix AM. Effect of L-3,4-dehydroproline on collagen synthesis and prolyl hydroxylase activity in mammalian cell cultures. J Biol Chem 1976;251:503–9.
[11] Rojkind M. Inhibition of liver fibrosis by L-azetidine-2-carboxylic acid in rats treated with carbon tetrachloride. J Clin Invest 1973;52:2451–6.
[12] Christensen HN, Cullen AM. Effects on non-metabolizable analogs on the distribution of amino acids in the rat. Biochim Biophys Acta 1968;150:237–52.
[13] Greenwel P, Schwartz M, Rosas M, Peyrol S, Grimaud JA, Rojkind M. Characterization of fat-storing cell lines derived from normal and CCl_4-cirrhotic livers: differences in the production of interleukin-6. Lab Invest 1991;65:644–53.
[14] Jimenez W, Parés A, Caballería J, Heredia D, Bruguera M, Torres M, Rojkind M, Rodés J. Measurement of fibrosis in needle liver biopsies: evaluation of a colorimetric method. Hepatology 1985;5:815–8.
[15] López-de León A, Rojkind M. A simple micromethod for collagen and total protein determination in formalin-fixed paraffin-embedded sections. J Histochem Cytochem 1985;33:737–43.
[16] Peterkofsky B, Diegelmann R. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 1971;10:988–94.
[17] Geerts A, Vrijsen R, Rauterberg J, Burt A, Schellinck P, Wisse E. *In vitro* differentiation of fat-storing cells parallels marked increase of collagen synthesis and secretion. J Hepatol 1989;9:59–68.
[18] Anania FA, Womack L, Potter JJ, Mezey E. Acetaldehyde enhances murine *α2(I)* collagen promoter activity by Ca^{2+}-independent protein kinase C activation in cultured rat hepatic stellate cells. Alcohol Clin Exp Res 1999;23:279–84.
[19] Herron GS, Banda MJ, Clark EJ, Gavrilovic J, Werb Z. Secretion of metalloproteinases by stimulated capillary endothelial cells. J Biol Chem 1986;261:2814–8.
[20] Theret N, Musso O, L'Helgoualc'h A, Clements B. Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. Am J Pathol 1997;150:51–8.
[21] Kilberg MS, Stevens BR, Novak DA. Recent advances in mammalian amino acid transport. Annu Rev Nutr 1993;13:137–65.
[22] Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJP. Mechanisms of spontaneous resolution of rat liver fibrosis. J Clin Invest 1998;102:538–49.
[23] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Current protocols in molecular biology. New York: Wiley, 1987.
[24] Maher J, Tzagaradis C, Gimenez A. Malondialdehyde stimulates collagen production by hepatic lipocytes only upon activation in primary culture. Alcohol Alcohol 1994;29:605–10.
[25] Freeman TL, Ngo HQ, Mailliard ME. Inhibition of system A amino acid transport and hepatocyte proliferation following partial hepatectomy in the rat. Hepatology 1999;30:437–44.
[26] Day CP. Is necroinflammation a prerequisite for fibrogenesis? Hepatogastroenterology 1996;43:104–20.
[27] Olaso E, Friedman SL. Molecular regulation of hepatic fibrogenesis. J Hepatol 1998;29:836–47.
[28] Junnila M, Rahko T, Sukura A, Lindberg LA. Reduction of carbon tetrachloride-induced hepatotoxic effects by oral administration of betaine in male Han–Wistar rats: a morphometric histological study. Vet Pathol 2000;37:231–8.
[29] Maezono K, Kajiwara K, Mawatari K, Shinkai A, Torii K, Maki T. Alanine protects liver from injury caused by D-galactosamine and CCl_4. Hepatology 1996;24:185–91.
[30] Carini R, Bellomo G, Grazia De Cesaris M, Albano E. Glycine protects against hepatocyte killing by KCN or hypoxia by preventing intracellular Na^+ overload in the rat. Hepatology 1997;26:107–12.