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Full Length Research Paper

Enalaprilat-induced cardiac fibroblast proliferation via regulation of transforming growth factor beta 1 (TGF-β1)-expressing anti-angiotensin II (Ang II)

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The objectives of this study were to investigate the effects of the angiotensin I-converting enzyme inhibitor enalaprilat (Ena) on the proliferation, hydroxyproline content, and transforming growth factor β1 (TGF-β1) protein expression of neonatal cardiac fibroblasts and to probe its anti-cardiac fibrosis mechanism. Cardiac fibroblast (CFb) was isolated using the trypsin digestion method. Methyl thiazolyl tetrazolium (MTT) colorimetric assay was performed to evaluate cell proliferation, a hydroxyproline method of determination was used to measure collagen content, and reverse transcription-polymerase chain reaction and flow cytometry were used to detect mRNA TGF-β1 and protein levels, respectively, with Ena. Ena decreased dramatically the MTT value, the hydroxyproline content, and inhibited TGF-β1 transcription and protein expression in neonatal rat CFb. The antiproliferative effects of Ena on CFb may be attributed to the inhibition of TGF-β1 transcription and protein expression in cardiac fibroblasts and the antagonistic action of angiotensin II (Ang II).

Key words: Enalaprilat, cardiac fibroblast, hydroxyproline, transforming growth factor-β_1.

INTRODUCTION

Myocardial fibrosis (MF) is characterized by cardiac fibroblast (CFb) excessive proliferation and collagen secretion, and it participates in ventricular remodeling. Myocardial fibrosis is a common pathological change of variety of cardiac disease develop to a certain stage, it is one of main performance of myocardial remodeling. Myocardial fibrosis shows the imbalance between the synthesis and degradation of extracellular matrix, interstitial collagen deposition increasing, the proportion of type I collagen imbalance, including type I, type III collagen ratio increasing, the disordered arrangement, etc. These pathological changes exist in many cardiovascular diseases, which are closely related with cardiac arrhythmia, cardiac dysfunction (Chen et al., 2004). MF fibrosis induced by angiotensin II (angiotnin II, Ang II), adrenergic, and endothelin can increase transforming growth factor beta 1 (TGF-β1). TGF-β1synthesis stimulated by Ang II promote occurrence of fibrosis, and increase TGF-β1 formation in endothelial cells and cardiac muscle synthesis of fibroblasts. TGF-β1contributes to the occurrence and progression of MF and its increased expression is closely related to MF (Hai and Zai 2007). Enalaprilat (Ena) lowers blood pressure, improves myocardial blood supply, and is used in the treatment of myocardial infarction; however, it also inhibits the proliferation of vascular smooth muscle cells and CFb (van Eickels et al., 2000).

Its mechanism of action on CFb thus needs to be further studied. In this study, we observed the effects of Ena on Ang II-induced CFb proliferation, hydroxyproline content, and CFb TGF-β1 transcription as well as expression at the cellular level to investigate its anti-MF mechanism.

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MATERIALS AND METHODS

Cells

The ventricles of Wistar rats (1 to 3 days old) were digested with trypsin, and the cells were collected, placed in Iscove's modified Dulbecco's medium (IMDM) containing 100 ml/L of fetal calf serum (FCS), and then cultured in 5% CO_2 at 37°C for 60 to 90 min. The CFb was separated using the differential adhesion method and then placed in IMDM containing 100 ml/L of FCS to culture. It was identified using inverted microscopy, transmission electron microscopy, and immunohistochemistry staining of fibronectin. The result was negative for smooth muscle actin staining. The purity of the CFb reached 98%, cultured nearly to fusion, and passage at 1:3. Second- to fourth-generation cells were used in the experiments.

Experimental groups

The cells were divided into the following groups: (1) the control group, which included the culture medium containing IMDM; (2) the Ang II group, containing the medium with the final concentration of 10^{-7} mol/L Ang II; (3) the Ena (10^{-5} mol/L) group, containing the final concentrations of 10^{-5} mol/L/Ena and 10^{-7} mol/L/Ang II; (4) the Ena (10^{-6} mol/L) group, containing the culture medium with 10^{-6} mol/L Ena and 10^{-7} mol/L Ang II; and (5) the Ena (10^{-7} mol/L/) group, containing the culture medium with 10^{-7} mol/L Ena and 10^{-7} mol/L Ang II.

Methyl thiazolyl tetrazolium (MTT) assay

The cells were seeded in 96-well culture plates, with eight cells per well, and were subjected to a drug intervention at 24, 48, and 72 h. Four hours before the end of the culture, 10 µl of 5 g/L MTT was added. Up to 150 µl of dimethyl sulfoxide was added after blue-violet crystalline precipitates were formed for degradation. Absorbance was measured at a wavelength of 490 nm (A_{490}) using enzyme-linked immunosorbent assay. Only the medium blank wells were adjusted to zero.

Determination of hydroxyproline

The groups were divided as they were for the MTT assay. A 24-well plate was used, and the drugs were added at 24, 48, and 72 h. The supernate containing CFb was taken as 1 ml, 200 µl per sample, the rest was stored at -20°C. The determination of hydroxyproline steps was as follows: 100 mg apical myocardial tissues were taken, washed with physiological saline and sheared, 1 ml hydrolyzate was added and mixed thoroughly, put into tube with glass cover, kept at 95°C or boiling for 20 min, the reagents that followed were added according to the kit's instruction, centrifuged at 3500 r/ min for 10 min, and the supernate was taken and the content of hydroxyproline was analyzed. The content of hydroxyproline value/0.134 was 1, the content of myocardial collagen protein.

Reverse transcription polymerase chain reaction (PCR)

Rat TGF-β1 cDNA sequences were obtained from GenBank (accession code X76881). The upstream and downstream primer sequences used were 5′-CTTCAGCTCCACAGAGAAGAACTGC-3′ and 5′-CACGATCATGTTGGACAACTGCTCC-3′, respectively. The sequences were complemented with mouse TGF-β cDNA, and the PCR product was 290 bp in size. In total, 5×10^4 ml^{-1} of CFb cells at logarithmic growth was taken. TRIzol was added to the cell lysate, and the total RNAs were extracted according to the manufacturer's instructions. PCR was carried out according to the standard procedure, and the PCR conditions were as follows: pre-denaturation for 5 min at 95°C and 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1.5 min at 72°C, followed by a final extension for 10 min at 72°C. The PCR products were detected using 2% agarose gel electrophoresis, ethidium bromide staining, and photography. The density of the amplified bands was analyzed using the gel analysis system. The average gray value represented the corresponding gene expression. β-Actin was used as an internal reference.

Flow cytometry

The cells were fixed, and 50 µl of specific anti-TGF-β1 was added to the medium; 0.1% phosphate buffered saline was used as control. The cells were incubated at 4°C overnight, washed twice with phosphate buffered saline, and then centrifuged. The supernatant was discarded to remove non-specific binding of the antibody. The staining dilution of the secondary antibody (fluorescein isothiocyanate/anti-mouse immunoglobulin G, 1:100) was added, and the supernatant was then incubated at 37°C in the dark for 45 min to 1 h. The solution was then filtered with 300-mesh nylon net and was detected. The amount of fluorescein isothiocyanate fluorescence excited was used to determine the level of cell TGF-β1 relative protein expression.

Statistical analysis

Data were expressed as mean ± standard deviation. All statistical analysis was performed using Statistical Package for Social Sciences (SPSS). Statistical significance was analyzed using analysis of variance (ANOVA), and intergroup comparisons were performed using *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemical identification

Under the microscope, cells were spindle-shaped or irregular triangle, cytoplasm was pale and almost transparent, refractive, and central oval nucleus was visible, cytoplasm protruding or ridged, cells arranged in a radial, pulsation-free (Figure 1A). Inverted microscope, immuno-cytochemical staining confirmed that cultured fibronectin cells was antibody-positive, monoclonal antibody against α-actin staining was negative (PBS as negative control). These results were in line with the dyeing characteristics of fibroblasts (Figure 1B and C).

After 24 h of treatment in each group, morphological changes in the cells were observed under an inverted microscope. The control cells had larger cell bodies, and the spindle-shaped cytoplasm as well as the connections between the cells was rich. In the Ang II group, cell proliferation was active and woven-like, the number of flame-shaped cell bodies increased, and cell growth was intensive. After Ena was added, cell density decreased, the cell gap increased, and the connections between the cells gradually decreased. The cells shrunk and became

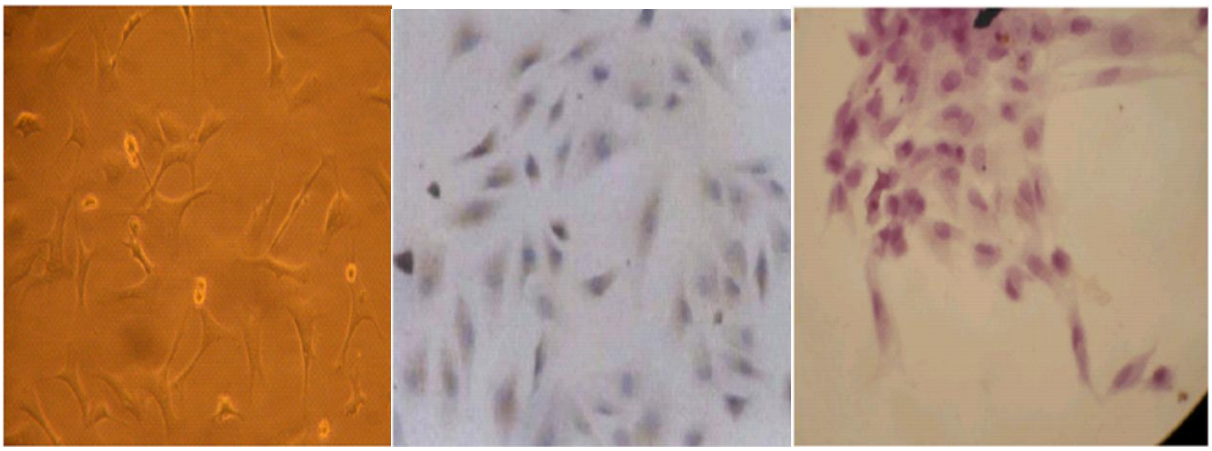

Figure 1. (A) Cardiac fibroblast in inverted microscope (24 h 200×); (B) Positive staining of Fibronectin in cardiac fibroblast (200×); (C) Negative staining of α-smooth muscle actin in cardiac fibrolast (200×).

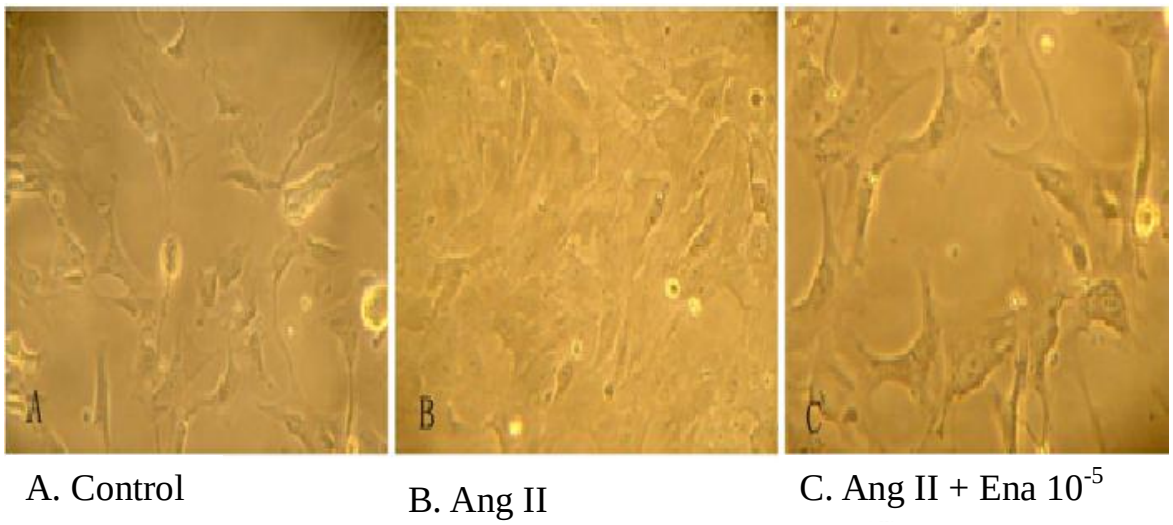

Figure 2. Effect of Ena on photo micrographs of CFb under microscope (×100).

mostly spindle-shaped or round, and light transmission was reduced (Figure 2).

Cell survive

Ang II significantly increased the metabolic rate of CFb to MTT. The MTT values significantly increased, and the difference was significant ($P < 0.01$) compared with the control group, suggesting that Ang II promotes cardiac fibroblast proliferation. After different concentrations of Ena were added, the MTT values in the control group became significantly lower than those in the Ang II group, with the difference being significant ($P < 0.05$), marking the dose-dependent impact of Ena (Table 1).

CFb collagen content

Significant differences ($P < 0.05$) in hydroxyproline content between the Ang II group and the control group were found. After treatment, the hydroxyproline content decreased. As Ena concentration increased, the dose-effect relationship became significant. Compared with the Ang II group, the changes in the Ena (10^{-5} mol/L) group at 24 and 48 h were somewhat significant ($P < 0.01$) and those at 72 h were significant ($P < 0.05$) (Table 2).

Reverse transcription PCR

As shown in Table 3 and Figure 3, TGF-β1 expression at

Table 1. Effect of Ena on CFb viability ($\bar{X}$ ± s, MTT-OD, n = 8).

Group	24 h	48 h	72 h
Control	0.2651 ± 0.0403	0.2543 ± 0.0653	0.1153 ± 0.0106
AngII	0.3000 ± 0.0383$^{\#}$	0.3449 ± 0.0203$^{\#\#}$	0.1820 ± 0.0207$^{\#}$
AngII+Ena10^{-7}	0.2308 ± 0.0636*	0.3290 ± 0.0304*	0.1706 ± 0.0150
AngII+Ena10^{-6}	0.2303 ± 0.0366*	0.2873 ± 0.0250*	0.1568 ± 0.0102
AngII+Ena10^{-5}	0.2280 ± 0.0428**	0.2206 ± 0.0560**	0.1402 ± 0.0145**

$^{\#}$, P < 0.05; $^{\#\#}$, P < 0.01 versus control; *, P < 0.05; **, P < 0.01 versus AngII.

Table 2. Effect of Ena on CFb collagen synthesis ($\bar{X}$ ±s, μg/mg, n = 8).

Group	24 h	48 h	72 h
Control	1.26 ± 0.36	1.74 ± 0.34	1.65 ± 0. 65
AngII	2.27 ± 0.54$^{\#}$	2.31 ± 0.35$^{\#}$	2.36 ± 0.20$^{\#}$
AngII+Ena10^{-7} mol/L	1.27 ± 0.57*	1.40 ± 0.37*	1.79 ± 0.56
AngII+Ena10^{-6} mol/L	1.21 ± 0.23**	1.36 ± 0.42*	1.72 ± 0.30*
AngII+Ena10^{-5} mol/L	1.15 ± 0.65**	1.28 ± 0.43**	1.67 ± 0.25*

$^{\#}$P<0.05, versus Control;*P<0.05,**P<0.01 versus AngII group.

Table 3. Effect of Ena on CFb TGF-β_1 mRNA in RT-PCR ($\bar{X}$ ±s, n=6).

Group	TGF-β_1/β-actin (relative density)
Control	0.35 ± 0.07
AngII	0.87 ± 0.21$^{\#}$
AngII+Ena10^{-7}	0.63 ± 0.16*
AngII+Ena10^{-6}	0.42 ± 0.07**
AngII+Ena10^{-5}	0.37 ± 0.06**

$^{\#}$P < 0.05 versus Control; *P < 0.05;**P < 0.01 versus AngII group.

the transcription level increased in the Ang II group when compared with the control group (P < 0.05). Three doses of Ena reduced the transcription of TGF-β1 in the corresponding groups, with the changes being significant when compared with the Ang II group (P < 0.05).

CFb TGF-β1 protein expression

As shown in Table 4, TGF-β1 protein expression in the Ang II group significantly increased when compared with the control group, with the difference being significant (P < 0.05).

The administration of three doses of Ena variably reduced the TGF-β1 protein expression in the corresponding groups when compared with the Ang II group (P < 0.05).

Correlation analysis of TGF-β1 mRNA and TGF-β1 protein expression

The TGF-β1 mRNA and TGF-β1 levels under different concentrations of Ena were compared and analyzed. The TGF-β1 mRNA expression and TGF-β1 protein levels revealed a significant positive correlation (r = 0.8089, P < 0.01). TGF-β1 protein content increased as TGF-β1 mRNA increases, indicating that Ang II can increase TGF-β1 synthesis at the transcription level and enhance TGF-β1 protein expression, thereby contributing to MF (Figure 4).

DISCUSSION

The TGF-β1 is a multifunctional protein peptide that can increase interstitial collagen-based protein synthesis, and it is one of the most common final intermediate factors of MF. It could increase mRNA expression of collagen types I and III in cells cultured *in vitro* (Devereux et al., 2002) and improve the mRNA stability of collagen I. The Ang II receptor has two variants: type 1 (AT1) and type 2 (AT2). Almost all AT receptors are mediated by the physiological functions and most of the pathological features of Ang II, highlighting the significance of blocking the AT1 receptor in the reversal of cardiac fibrosis (Maria et al., 2001). Converting enzyme inhibitors are the first drug to be used in clinical studies among selective non-peptide Ang II receptor antagonists. They have a direct role in the AT1 receptor, which can control high blood pressure, reduce

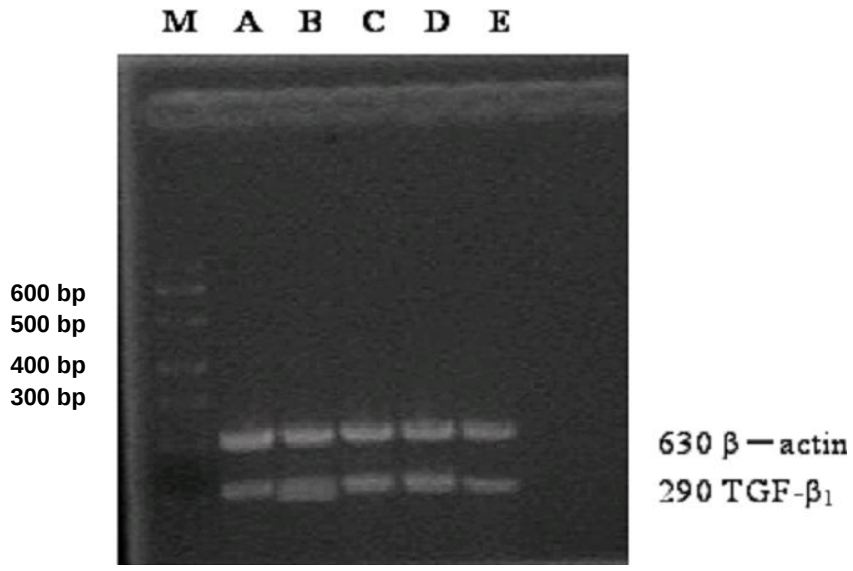

Figure 3. Effect of Ena on CFb TGF-β1 protein expression detected by RT-PCR. M: DNA Marker; A: Control; B: Ang II; C: Ang II+Ena 10^{-7}; (D) Ang II+Ena10^{-6}; (E) Ang II + Ena10^{-5}.

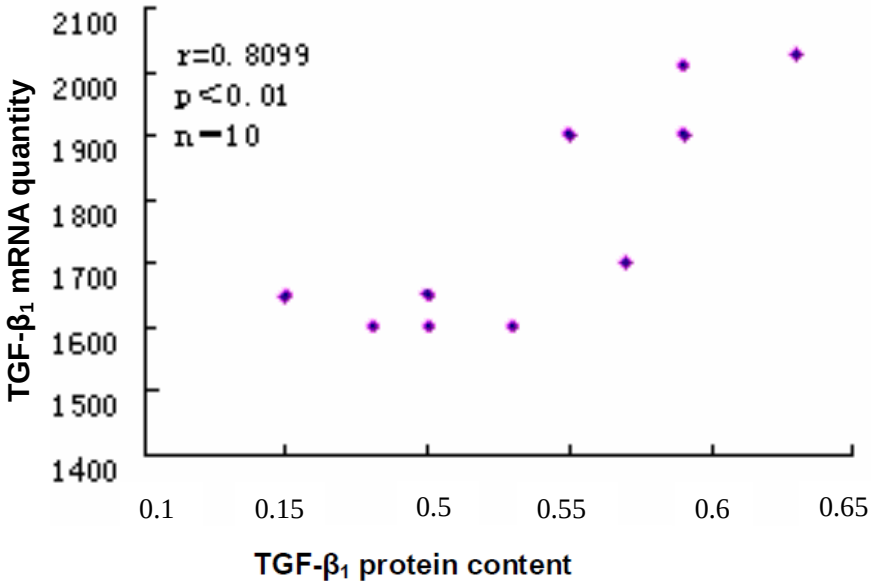

Figure 4. Relativity between TGF-β_1 mRNA quantity and TGF-β_1 protein content.

Table 4. Effect of Ena on CFb TGF-β_1 protein expression in FCM ($\bar{X}$ ±s, n=6).

Group	TGF-β_1 (10,000 cells)
Control	1514 ± 366
AngII	2025 ± 257#
AngII+Ena10^{-7}	1810 ± 345*
AngII+Ena10^{-6}	1726 ± 284*
AngII+Ena10^{-5}	1646 ± 243**

#, P < 0.05 versus control; *, P < 0.05; **, P < 0.01 versus Ang II. group.

proteinuria, and control the expression of local cytokines in heart and kidney tissues, such as platelet-derived growth factor and TGF-β1, to delay heart and kidney fibrosis (Iwanciw et al., 2003).

Cardiac interstitial MF is the excessive proliferation of fibroblasts and excessive deposition of collagen characterized by abnormal distribution of interstitial cardiac remodeling (Nadal et al., 2002). By acting on CFb, the Ang II AT1 receptor causes CFb proliferation and collagen synthesis, which in turn lead to MF. Our results show that Ena administration, under the action of

Ang II, significantly reduced CFb MTT-optical density (OD) and collagen content ($P < 0.05$).

Recent studies found out that TGF-β1 and Ang II have closely related roles in MF: Ang II increases TGF-β1 gene expression, whereas TGF-β1 inhibits extracellular matrix degradation and increases extracellular matrix mRNA expression and protein synthesis (Laviades et al., 2006). TGF-β1 has an important role in cell proliferation, differentiation, migration, and deposition of the extracellular matrix in the myocardium. TGF-β1 receptors are distributed in cardiac myocytes and non-myocardial cells (Engelmann and Grutkoski, 2004). Experiments have shown that receptors I to III of TGF-β1 exists on the surface of neonatal rat cardiac myocytes (Roberts et al., 2007). TGF-β1 receptors in cardiac fibroblasts have also been reported (Li et al., 2005). Wang et al. (2007) found that TGF-β1 can stimulate collagenase and collagenase promoter activity in cardiac fibroblasts. Other researchers suggested that angiotensin II induces extracellular matrix synthesis through TGF-β1 (Sadoshima and Izumo, 1993).

Ena is the active form of enalapril that can inhibit CFb proliferation and collagen synthesis (Sun and Yang 2009). However, data about the changes in TGF-β1 mRNA and protein expression under the intervention of Ena and their relationship with MF are limited. This study determined that TGF-β1 mRNA and protein expression significantly increased in the Ang II group. After treatment with Ena, the TGF-β1 mRNA and protein expression of each dose group reduced to varying extents, and the differences were significant when compared with the Ang II group ($P < 0.05$).

The correlation analysis showed that a significant positive correlation existed at the level of TGF-β1 transcription and protein expression ($r = 0.8099$, $P < 0.01$), which indicates that the increased TGF-β1 expression was caused by the increased transcription activity. Ena inhibits TGF-β1 protein expression by inhibiting CFb TGF-β1 transcription (Chin et al., 2001). Animal experiments and *in vitro* cell culture studies have shown that angiotensin-converting enzyme inhibitors (ACEI) treatment can reduce collagen synthesis (Sasaguri et al., 2000) and prevent as well as reverse cardiac fibrosis. Much research has demonstrated that the activation of factors of collagen synthesis in cardiac collagen deposition and fibrosis, including Ang II, ALD, ET, TGF-β1, and CTGF, among others, could block the activation of the neuroendocrine system, rendering them key factors in the reversal of MF (Li and Fan, 2004; Zhang et al., 2004).

Conclusively, the inhibition of extracellular matrix degradation by TGF-β1 could promote the occurrence of MF-induced collagen synthesis. Moreover, the inhibition of TGF-β1 transcription and protein expression by Ena dose-dependently reduces collagen synthesis and secretion, lowers the biological activity of Ang II, and inhibits CFb proliferation as well as collagen synthesis, thus inhibiting MF (Gruden et al., 2000; Rona et al., 1995).

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