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Title: A novel chymase inhibitor, BCEAB (4-[1-{[bis-(4-methyl-phenyl)-methyl]-carbamoyl}-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid) suppressed cardiac fibrosis in cardiomyopathic hamsters

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Running title: Role of chymase in cardiomyopathy

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Abbreviations: TGF-β**,** transforming growth factor-β; ACE, angiotensin-converting enzyme; ARB, angiotensin II type 1 receptor blocker

Abstract

Previously, we reported that levels of chymase activity and its mRNA in cardiac tissues were significantly increased along with progression of cardiac fibrosis in cardiomyopathic hamsters, but the involvement of chymase in the progression of fibrosis has been unclear. In cultured human fibroblasts, the concentration of transforming growth factor-β in the supernatant of media was significantly increased after injection of human chymase. Furthermore, human chymase dose-dependently increased cell proliferation, and this chymase-dependent proliferation was completely suppressed by a chymase inhibitor, Suc-Val-Pro-Phe^{p}(OPh)$_2$ (10 μM), or an anti-transforming growth factor-β antibody (100 μg/ml). In this study, we used Bio14.6 and F1B hamsters as cardiomyopathic and control hamsters, respectively. Cardiomyopathic hamsters were orally administered a novel chymase inhibitor, 4-[1-{[bis-(4-methyl-phenyl)-methyl]-carbamoyl}-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid (BCEAB, 100 mg/kg per day) or placebo from 5-week-old to 45-week-old. In the placebo-treated group, the cardiac chymase activity in cardiomyopathic hamsters aged of 45 weeks was significantly increased compared with that in control hamsters. BCEAB significantly reduced the cardiac chymase activity. The indexes (+dP/dt and -dP/dt) of cardiac function were significantly improved by treatment with BCEAB. The mRNA levels of collagen I and collagen III in the placebo-treated hamsters were significantly reduced to 69.6% and 76.5% by treatment with BCEAB, respectively. The fibrotic area in cardiac tissues in the BCEAB-treated hamsters was significantly suppressed to 50.7% compared with that in the placebo-treated treated hamsters. Therefore, the activation of transforming

growth factor-β by chymase may play an important role in the progression of cardiac fibrosis and cardiac dysfunction in cardiomyopathy.

Keywords: Chymase, Cardiomyopathy, Cardiovascular, Transforming growth factor-β, Fibrosis, Angiotensin II

In cardiac tissue of cardiomyopathic patients and hamsters, transforming growth factor (TGF)-β, which is a well-known growth factor that stimulates fibrosis via the accumulation of extracellular matrix, is increased (Eghbali et al., 1991; Li et al., 1997). Mast cells are found in increased numbers in the myocardial fibrotic area in cardiomyopahy, and this increase of cardiac mast cells may contribute to the development of fibroblast proliferation in cardiac tissues of cardiomyopathy (Panizo et al., 1995; Patella et al., 1998). However, mast cells release a large number of inflammatory mediators such as histamine, serotonin, chemotactic factors, cytokines and serine proteases during inflammation and repair process (Sperr et al., 1994; Marone et al., 1995). It has been unclear which factor plays an important role in the fibroblast proliferation of cardiac tissues.

Chymase is a chymotrypsin-like serine protease contained in the secretory granules of mast cells. Chymase has been known to activate angiotension I to form angiotensin II, and this enzyme may also contribute to the activation of TGF-β (Takai et al., 1996, 1999; Taipale et al., 1995). TGF-β is released from a latent TGF-β-binding protein in fibroblasts (Kanzaki et al., 1990). The latent TGF-β-binding protein is cleaved as latent TGF-β, and the latent form of TGF-β is activated to TGF-β by extremes of pH and by plasmin (Lawrence et al., 1985; Lyons et al., 1990; Miyazono and Heldin, 1989). Taipale et al. (1995) suggested that chymase could contribute to the release of latent TGF-β from latent TGF-β-binding proteins of the extracellular matrix of human epithelial and endothelial cells. However, it has been unclear whether chymase directly converts a latent TGF-β-binding protein of fibroblasts to an active form of TGF-β that can stimulate

cell proliferation of fibroblasts, and whether its function can contribute to the progression of cardiac fibrosis in cardiomyopathy.

Here, we clarified that chymase functions as a TGF-β-activating enzyme in human fibroblasts and investigated the effect of a specific chymase inhibitor on the progression of cardiac fibrosis and cardiac dysfunction in cardiomyopathic hamsters.

Methods

In vitro experiments

Materials

Human dermal fibroblasts (passage 4) and CS-C media kits were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). Purified human chymase was obtained, as described previously (Takai et al., 1997). A chymase inhibitor, Suc-Val-Pro-Phe^{P}(OPh)$_2$ was a gift from Dr. Oleksyszyn (Oleksyszyn and Powers, 1991). An angiotensin II type 1 receptor blocker (ARB), HR720, was a gift from Nippon Roussel (Tokyo, Japan). TGF-β was obtained from Chemicon International (Temecula, CA). Anti-TGF-β neutralizing antibody, latent TGF-β and an ELISA kit for measurement of TGF-β concentration were obtained from R & D Systems (Minneapolis, MN). Flasks and 96-well plates were purchased from Iwaki Glass (Tokyo, Japan). Trypsin, penicillin and streptomycin were purchased from Gibco (Rockville, MD).

Cell culture

In the preparation of experiments for determination of cell counts, the cells were grown to subconfluence. After the cells reached 80% confluence, the fibroblasts were seeded at a density of 5 x 10^4 cells/cm^2 and cultured for 48 h in serum-free CS-C medium. The quantity of TGF-β in the media supernatants was determined by ELISA 30, 60, 120 and 180 min after the injection of human chymase (25 ng/ml) or human chymase (25 ng/ml) plus Suc-Val-Pro-Phe^{P}(OPh)$_2$ (10 μM) into

a well containing the cultured fibroblasts.

After the cells reached 80% confluence, the fibroblasts were seeded at a density of 5 x 10^4 cells/cm^2 and cultured for 48 h in serum-free CS-C medium. The media were changed to serum-free CS-C medium supplemented with insulin and transferrin and were incubated for 24 h in the presence of human chymase (6.25 - 25 ng/ml), Suc-Val-Pro-$Phe^P(OPh)_2$ (10 μM), HR720 (1 μM), anti-TGF-β neutralizing antibody (100 μg/ml), TGF-β (100 – 500 pg/ml) or latent TGF-β (100 – 500 pg/ml). The cell proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation (Brd U detection kit III, Roche Diagnostics Co., Indianapolis, IN).

***In vivo* experiments**

Drugs and animals

A novel chymase inhibitor, BCEAB (4-[1-{[bis-(4-methyl-phenyl)-methyl]-carbamoyl}-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid), was a gift from Shionogi Co. (Toyonaka, Osaka, Japan). The cDNA probes used were as follows: rat collagen I cDNA (a 1.3-kb *Pst*I/*Bam*HI fragment) (Genovese et al., 1984); mouse collagen III cDNA (a 1.8-kb *Eco*RI/*Eco*RI fragment) (Liau et al., 1985); rat GAPDH (a 1.3-kb *Pst*I/*Pst*I fragment) (Fort et al., 1985).

Male cardiomyopathic Syrian hamsters (Bio 14.6) (5 weeks old; Charles River Breeding Laboratories, Tokyo, Japan) and age-matched control hamsters (F1B) (Charles River Breeding Laboratories) were used. BCEAB at a dose of 100 mg/kg per day or placebo was orally administered to animals staring from 5 weeks of age to 45 weeks. BCEAB was mixed in feed for administration. The body weight and

weight of feed eaten per day were measured once a week. Based on measurement results, doses of BCEAB to be mixed in feed were adjusted every week. The experimental procedures for animals were conducted in accordance with the guidelines of Osaka Medical College. The hamsters were fed regular chow, had free access to tap water, and were housed in a temperature-, humidity-, and light-controlled room.

Hemodynamic measurement

Hemodynamic measurements were performed in 45-week-old hamsters. A polyethylene catheter was introduced into the right carotid artery, and the mean arterial blood pressure and heart rate were measured (Jin et al., 2002). After this procedure, the thorax was opened under positive-pressure respiration and a catheter was inserted into the left-ventricle chamber via its apex, where left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), as well as maximal positive and negative rates of pressure development (+dP/dt and -dP/dt) were measured (Jin et al., 2002). At the end of the experiment, trunk blood and heart samples were harvested for later biochemical assays and histological assessments.

Measurements of enzyme activities

The heart was homogenized in 10 volumes (w/v) of 20 mM Na-phosphate buffer, pH 7.4. The homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was discarded, and the pellet was re-suspended and homogenized in 5 volumes (w/v) of 10 mM Na-phosphate buffer, pH 7.4, containing 2 M KCl and

0.1% Nonidet P-40. The homogenate was stored overnight at 4°C, and centrifuged at 20,000 x g for 30 min. The supernatant was used for measurement of the chymase and ACE activities.

The chymase activity was measured by incubating tissue extracts for 30 min at 37°C with 4 mM angiotensin I in 150 mM borax-borate buffer, pH 8.5, containing 8 mM dipyridyl, 770 µM diisopropyl phosphorofluoridate, which could not inhibit hamster chymase at this concentration (Takai et al., 1996), and 5 mM ethylenediaminetetraacetic acid, as described previously (Jin et al., 2002). The reaction was terminated by addition of 15% trichloroacetic acid, and then the mixture was centrifuged at 20,000 x g for 5 min at 4°C. One unit of chymase activity was defined as the amount of enzyme that cleaved 1 µmol angiotensin I/min. A blank was also included, the addition of 500 µM chymostatin.

The ACE activity was measured using a synthetic substrate, hippuryl-His-Leu (Peptide Institute, Inc., Osaka, Japan), specifically designed for ACE. The tissue extract or plasma was incubated for 30 min at 37°C with 5 mM hippuryl-His-Leu in 10 mM phosphate buffer, pH 8.3, containing 600 mM NaCl (Jin et al., 2002). The reaction was terminated by addition of 3% metaphosphoric acid, and then the mixture was centrifuged at 20,000 x g for 5 min at 4°C. The supernatant was applied to a reversed-phase column (4 mm i.d. x 250 mm, IRICA Instrument, Kyoto, Japan), which had been equilibrated with 10 mM KH_2PO_4 and methanol (1:1, pH 3.0), and eluted with the same solution at a rate of 0.3 ml/min. One unit of ACE activity was defined as the amount of enzyme that cleaved 1 µmol hippuric acid/min.

Protein concentration was assayed with BCA Protein Assay Reagents (Pierce, Rockford, IL), using bovine serum albumin as a standard.

Assessment of fibrotic area and mast cell number

After trunk blood collection, the hearts were collected. For the determination of fibrotic area, four transverse slices approximately 3 mm thick were cut from apex to base in the other hearts and these slices were fixed in methanol-Carnoy's fixative and embedded in paraffin. Four 5-μm sections were cut from each slice. Every section from the 4 slices was stained with azan stain and the blue area stained with azan stain was determined as fibrotic area. The ratio of fibrotic area to total cardiac area was measured by using a computerized morphometry system, MacSCOPE Ver 2.2 (Mitani Co., Fukui, Japan). The mast cells in every section from the 4 slices were stained with toluidine blue, and the number of mast cells was quantified, using the computerized morphometry system, and expressed as the number of stained mast cells per mm^2.

RNA isolation and Northern blot hybridization

Total RNA was extracted from the heart, as previously described (Kim et al., 1994). The RNA concentration was spectrophotometrically determined at 260 nm. Twenty micrograms of total RNA from the heart were denatured by incubation with 1 M deionized glyoxal and 50% dimethyl sulfoxide at 50°C for 1 h, electrophoresed on a 1% agarose gel at 50 V, and transferred to a nylon membrane (Gene Screen Plus, DuPont-Merck, Boston, MA) (Kim et al., 1994). Each cDNA probe was labeled with ^{32}P-deoxycytidine 5'-triphosphate (specific activity, 3,000 Ci/mM; DuPont) by the random primer extension method with a Random Primer DNA Labeling Kit (Takara Shuzo Co., Shiga, Japan). Prehybridization and hybridization were

performed according to the manufacturer's instructions, as previously described (Kim et al., 1994). To evaluate tissue mRNA levels, we used an optical scanner (EPSON GT8000, Seoko, Tokyo, Japan) to digitize the autoradiograms. The densities of the autoradiogram bands in the digitized image were measured with the use of the public-domain National Institutes of Health Image Program, as previously described (Kim et al., 1994). For all RNA samples, the density of an individual mRNA band was normalized to that of GAPDH to correct for differences in RNA loading and/or transfer.

Statistical analysis

All numerical data shown in the text are expressed as the mean ± standard error of the mean (SEM). Significant differences between the mean values of two groups were evaluated by Student's *t*-test for unpaired data. Significant differences among the mean values of multiple groups were evaluated by 1-way ANOVA followed by a post-hoc analysis (Fisher's test). $P < 0.05$ was used as the threshold for statisticaly significant differences.

Results

In vitro experiments

The concentration of TGF-β in the supernatant of media in cultured fibroblasts was significantly increased starting from 10 min after injection of human chymase. (Fig. 1).

To investigate the direct effect of chymase in fibroblasts, we initially focused on the cell proliferation of human fibroblasts using BrdU incorporation. In human fibrobrasts, chymase significantly increased the BrdU incorporation, and this increased BrdU incorporation was suppressed by a chymase inhibitor, Suc-Val-Pro-Phe^{P}(OPh)$_2$ and anti-TGF-β neutralizing antibody but not by an ARB, HR720 (Fig. 2). On the other hand, the BrdU incorporation in human fibrobrats could be induced by TGF-β, but not by latent TGF-β (Fig. 3).

In vivo experiments

Significant differences in body weight and ratio of heart weight to body weight were not observed at 45 weeks of age in cardiomyopathic hamsters treated with placebo or a chymase inhibitor (BCEAB) (Fig. 4). On the other hand, the mean arterial pressure and the +dP/dt, -dP/dt and LVSP, but not the LVEDP, were significantly improved by treatment with BCEAB (Fig. 4).

Plasma ACE activity was not affected by treatment with BCEAB (Fig. 5). In the cardiac tissues, chymase activity was significantly suppressed by treatment

with BCEAB ($P < 0.01$), while ACE activity was not (Fig. 5).

The mRNA levels of collagen I and collagen III in the placebo-treated hamsters were significantly reduced to 69.6% and 76.5% by treatment with BCEAB, respectively (Fig. 6).

Typical photographs of hearts of cardiomyopathic hamsters treated with placebo or BCEAB, stained with azan stain are shown in Figure 7. The fibrotic area in cardiac tissues in the BCEAB-treated hamsters was significantly suppressed to 50.7% compared with that in the placebo-treated hamsters. (Fig. 7, $P < 0.01$). The number of mast cells per mm^2 was significantly increased in the hearts of cardiomyopathic hamsters (F1B hamsters, 0.312 ± 0.08; Placebo-treated cardiomyopathic hamsters, 1.96 ± 0.32). In the BCEAB-treated hamsters, the number of mast cells per mm^2 tended to be decreased in the hearts of cardiomyopathic hamsters (1.49 ± 0.24).

Discussion

To clarify the existence of chymase-dependent TGF-β activation, we used human dermal fibroblasts, which has no response against angiotensin II (Pun et al., 1988), to remove the contribution of angiotensin II, because chymase is known to convert angiotensin I to angiotensin II, which can induce cell proliferation of other fibroblasts (Nakamura et al., 1994; Taniyama et al., 2000). In the present study, chymase was found to a significantly increase cell proliferation in fibroblasts, but this increased cell proliferation was completely suppressed by a chymase inhibitor, but not by an ARB. In media supernatants of the cultured fibroblasts, the concentration of TGF-β protein was significantly increased after the injection of chymase, but this increase of TGF-β was inhibited by a chymase inhibitor. Anti-TGF-β neutralizing antibody completely suppressed the cell proliferation induced by human chymase, indicating that chymase induced the cell proliferation through TGF-β activation. On the other hand, TGF-β could induce the cell proliferation in human fibroblasts, but latent TGF-β could not. These findings suggested that chymase not only can convert latent TGF-β-binding proteins in fibroblasts to latent TGF-β but can also activate the latent TGF-β (inactive form) to active TGF-β (Fig. 8). Therefore, cell proliferation of fibroblasts may be directly induced by chymase, which represents an autocrine factor sustaining TGF-β activation in fibroblasts.

In the cardiomyopathic hamster, expression of collagen I and collagen III genes were significantly increased in cardiac tissues (Dixon et al., 1997). Increased collagen synthesis may play an important role in impairing cardiac function in the development of cardiomyopathy. TGF-β is known to induce the expression of collagen I and collagen III genes (Lijnen et al., 2000). Recently, Kuwahara et al. (2002) reported that in pressure-overloaded rats, the administration of anti-TGF-β neutralizing antibody prevented both the expression of collagen genes and myocardial fibrosis, but not myocyte hypertrophy. In the present study, a chymase inhibitor suppressed expression of collagen I and collagen III genes in cardiac tissues and reduced the fibrotic area, but not the ratio of heart weight to body weight. These findings might be very similar to the results of anti-TGF-β neutralizing antibody treatment. Therefore, the increase of cardiac chymase activity in cardiomyopathy may induce TGF-β activation, and its function may play an important role in inducing cardiac fibrosis and dysfunction via induction of the expression of collagen I and collagen III genes. In the present study, the +dP/dt, -dP/dt and LVSP were significantly improved by treatment with BCEAB. However, LVEDP did not change by treatment with the chymase inhibitor, inferring that there was no significant difference in LVEDP between normal and cardiomyopathic hamsters, and diastolic function might not be markedly damaged at that time. Long-term studies are needed to observe the diastolic dysfunction.

Gene expression of TGF-β is known to be induced by angiotensin II (Nakamura et al., 1994; Taniyama et al., 2000). Chymase can produce angiotensin II from angiotensin I (Takai et al., 1996; Takai et al., 1999), and this angiotensin II

produced by chymase may be involved in the pathogenesis of cardiac fibrosis in cardiomyopathic hamsters. In fact, ARB could prevent fibrotic formation in cardiomyopathic hamsters (Nakamura et al., 1994; Taniyama et al., 2000). ARB reduced the ratio of heart weight to body weight in cardiomyopathic hamsters in addition to reducing the expression of collagen genes and cardiac fibrosis (Nakamura et al., 1994; Taniyama et al., 2000), while a chymase inhibitor did not affect the ratio of heart weight to body weight in the present study. The difference between ARB and chymase inhibitor relative to the effect on the ratio of heart weight to body weight suggests different mechanisms in their improvement of cardiac function.

In the present study, we used BCEAB, which was developed recently as an orally active specific chymase inhibitor (Takai et al., 2001). The cardiac chymase activity in normal hamsters was significantly reduced by oral, once-daily administration of 100 mg/kg of BCEAB, and this dose of BCEAB was chosen in the present study (Takai et al., 2001). In the present study, the number of mast cells per mm^2 of the hearts in the placebo-treated cardiomyopathic hamsters was significantly higher than in the normal hamsters, while it tended to be decreased in the BCEAB-treated hamsters. It is known that chymase plays an important role in the accumulation of mast cells by activating stem cell factor (Zhang et al., 1998). Hara et al. (2002) reported that cardiac fibrosis decreased significantly in the hearts of mast cell deficient mice compared to those of normal mice. Cardiac hypertrophy in the model was suppressed significantly by mast cell stabilizers. The findings suggest that mast cells play an important role in cardiac remodeling. Therefore, decreases in chymase activity by the chymase inhibitor in the heart might be associated with decreases in mast cells, in addition to direct suppression.

In the hamster uterus-injured adhesion model, the chymase activity was significantly increased in the adhesion site after injury, while it was significantly reduced by treatment with BCEAB, at the dose same (100 mg/kg per day) to that used in the present experiments, along with the reduction of adhesion formation which is closely related to fibrotic formation (Okamoto et al., 2002). Furthermore, in this adhesion model, the TGF-β concentrations in the peritoneal fluid were significantly increased after scraping the uterus, while the increased TGF-β concentrations were also reduced by treatment with BCEAB (100 mg/kg per day) (Okamoto et al., 2002). Therefore, chymase inhibition might be related to the reduction of TGF-β activation *in vivo*.

In this study, we demonstrated that chymase could directly activate a latent TGF-β-binding protein in fibroblasts and that a novel chymase inhibitor, BCEAB, was useful for preventing progression of cardiac fibrosis in cardiomyopathic hamsters. The activation of TGF-β by chymase may play an important role in the progression of cardiac fibrosis in cardiomyopathy.

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Figure legends

Figure 1 The quantity of TGF-β in the media supernatants was determined by ELISA 30, 60, 120 and 180 min after the injection of human chymase (25 ng/ml) or human chymase (25 ng/ml) plus Suc-Val-Pro-Phe^{P}(OPh)$_2$ (10 μM) into a well containing the cultured fibroblasts. Values are means ± SEM (n = 6). $^{**}P < 0.01$ vs. injection of huma chymase plus Suc-Val-Pro-Phe^{P}(OPh)$_2$.

Figure 2 Effects of human chymase (6.25 – 25 ng/ml) or human chymase (25 ng/ml) plus Suc-Val-Pro-Phe^{P}(OPh)$_2$ (CHI, 10 μM), HR720 (ARB, 1 μM) or anti-TGF-β neutralizing antibody (ATA, 100 μg/ml) in the cell proliferation of human fibroblasts. Values are means ± SEM (n = 6). $^{**}P < 0.01$ vs. injection of human chymase (25 ng/ml).

Figure 3 Effects of latent TGF-β or TGF-β in the cell proliferation of human

fibroblasts. Values are means ± SEM (n = 6). $^{**}P < 0.01$ vs. control.

Figure 4 Effects of a chymase inhibitor, BCEAB, on body weights, heart weights and hemodynamic measurements in 45-week-old cardiomyopathic hamsters. Values are means ± SEM (n = 8). $^{*}P < 0.05$ vs. placebo-treated group.

Figure 5 Effects of a chymase inhibitor, BCEAB, on enzyme activities of plasma ACE and cardiac chymase and ACE in 45-week-old cardiomyopathic hamsters. Values are means ± SEM (n = 8). $^{**}P < 0.01$ vs. placebo-treated group.

Figure 6 Effects of a chymase inhibitor, BCEAB, on expression profile (A) and mRNA levels (B) of collagen I and collagen III in 45-week-old cardiomyopathic hamsters. F, P and C represent F1B hamsters and placebo- and chymase inhibitor-treated cardiomyopathic hamsters, respectively (A). Values are means ± SEM (n = 8). $^{**}P < 0.01$ vs. placebo-treated group.

Figure 7 Effects of a chymase inhibitor, BCEAB, on the cardiac fibrosis in 45-week-old cardiomyopathic hamsters. Typical photographs of hearts of cardiomyopathic hamsters treated with placebo (left) or BCEAB (right), stained with azan stain (A). Arrows show the fibrotic lesions (blue) stained with azan stain (A). The ratio of fibrotic area to total cardiac area in 45-week-old cardiomyopathic hamsters (B). Values are means ± SEM (n = 8). $^{**}P < 0.01$ vs. placebo-treated group.

Figure 8 Role of human chymase on TGF-β activation from human fibroblasts.

Fig. 1

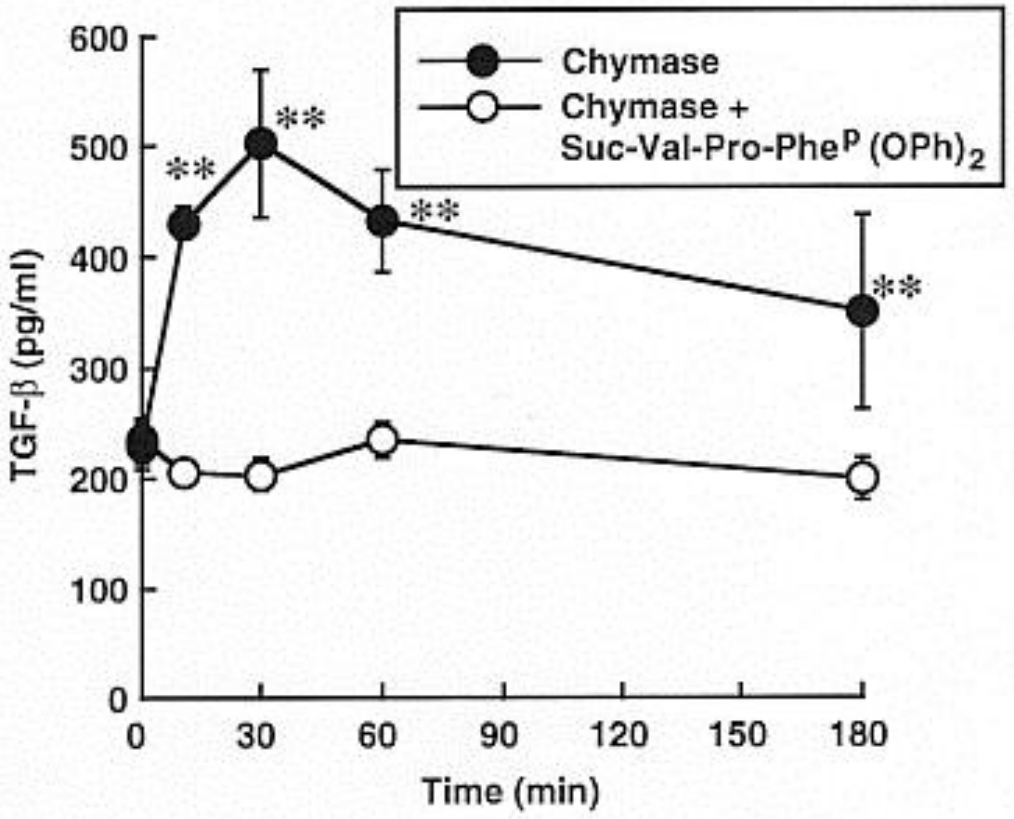

Chymase
Chymase +
Suc-Val-Pro-Phe^{P} $(OPh)_2$
TGF-β (pg/ml)
600
500
400
300
200
100
0
0
30
60
90
120
150
180
Time (min)
**
**
**
**

Fig. 2

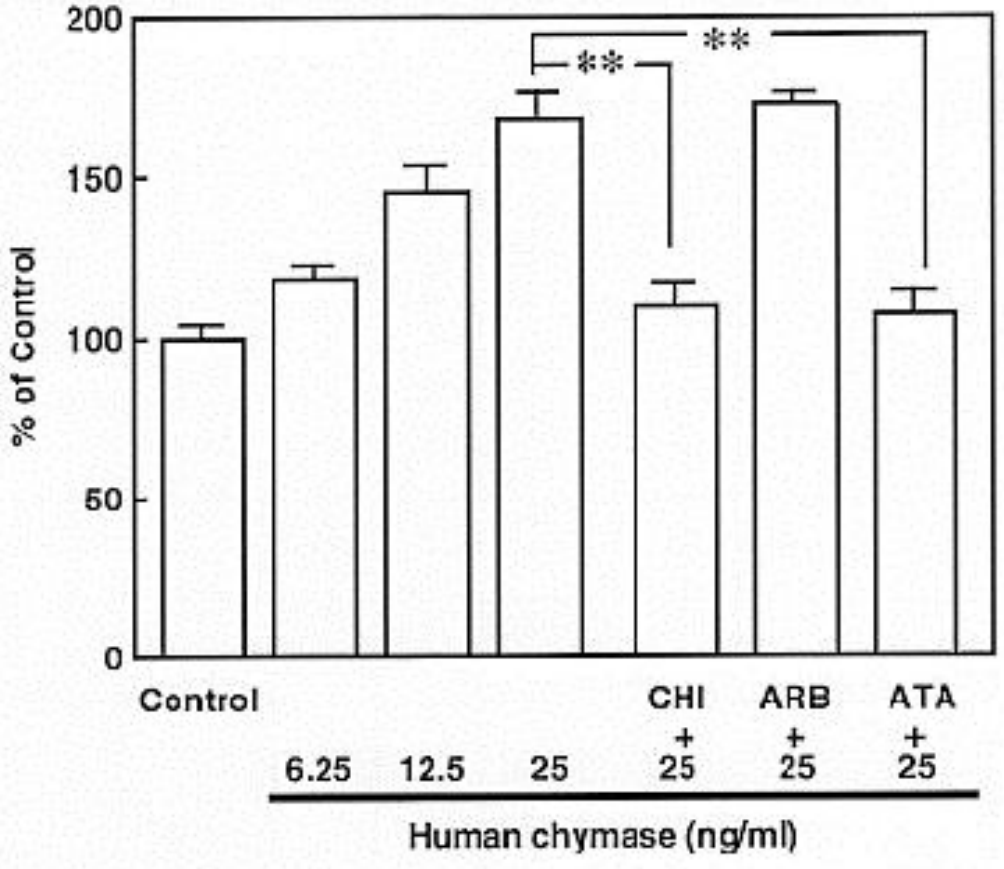

200
150
100
50
0
% of Control
**
**
Control
6.25
12.5
25
CHI + 25
ARB + 25
ATA + 25
Human chymase (ng/ml)

Fig. 3

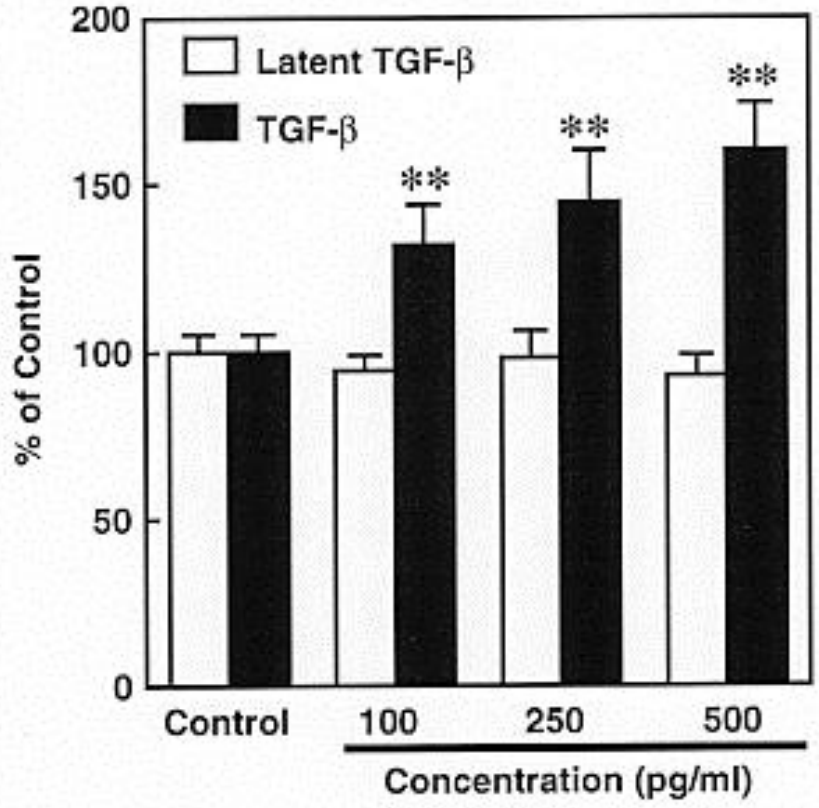

200
150
100
50
0
% of Control
Latent TGF-β
TGF-β
**
**
**
Control
100
250
500
Concentration (pg/ml)

Fig. 4

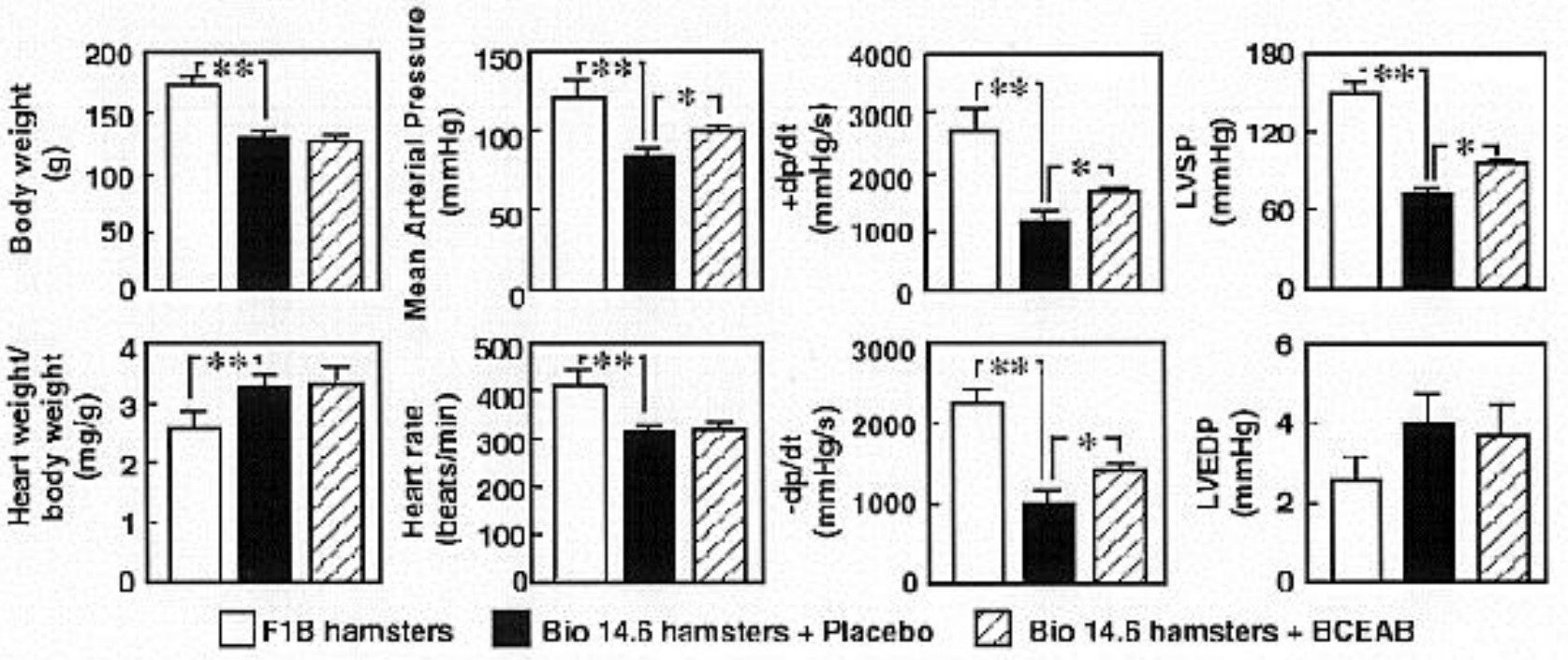

Body weight (g)
Mean Arterial Pressure (mmHg)
+dp/dt (mmHg/s)
LVSP (mmHg)
Heart weight/ body weight (mg/g)
Heart rate (beats/min)
-dp/dt (mmHg/s)
LVEDP (mmHg)
F1B hamsters
Bio 14.6 hamsters + Placebo
Bio 14.6 hamsters + BCEAB

Fig. 5

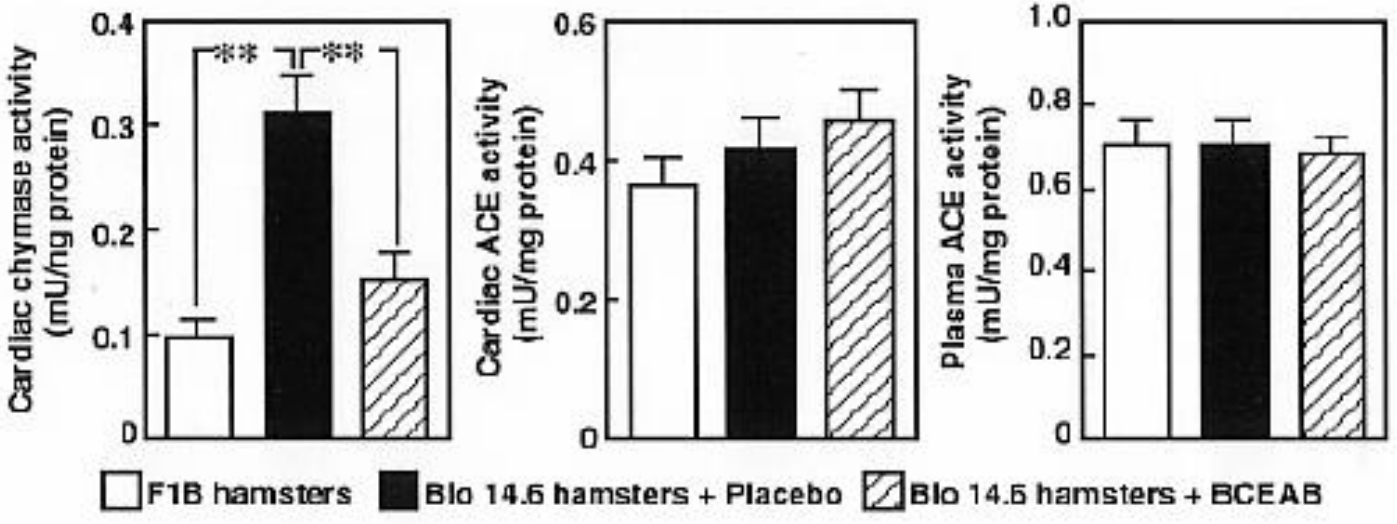

Cardiac chymase activity (mU/ng protein)
0.4
0.3
0.2
0.1
0
**
**
Cardiac ACE activity (mU/mg protein)
0.6
0.4
0.2
0
Plasma ACE activity (mU/mg protein)
1.0
0.8
0.6
0.4
0.2
0
F1B hamsters
Bio 14.6 hamsters + Placebo
Bio 14.6 hamsters + BCEAB

Fig. 6

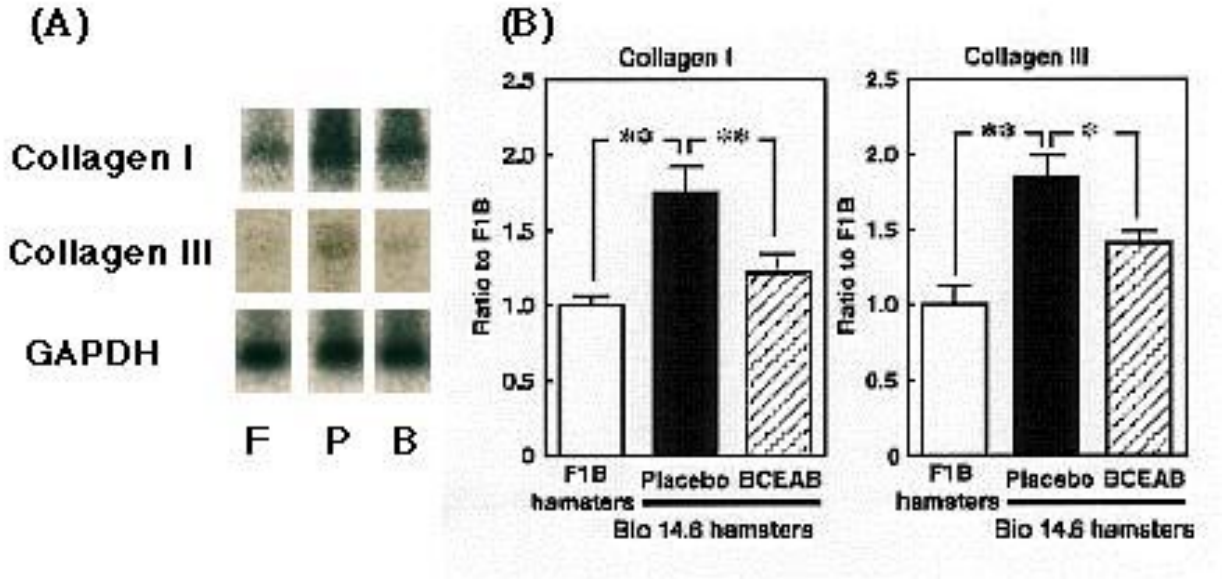

(A)
Collagen I
Collagen III
GAPDH
F
P
B
(B)
Collagen I
Collagen III
Ratio to F1B
2.5
2.0
1.5
1.0
0.5
0
**
**
*
F1B hamsters
Placebo
BCEAB
Bio 14.6 hamsters

Fig. 7

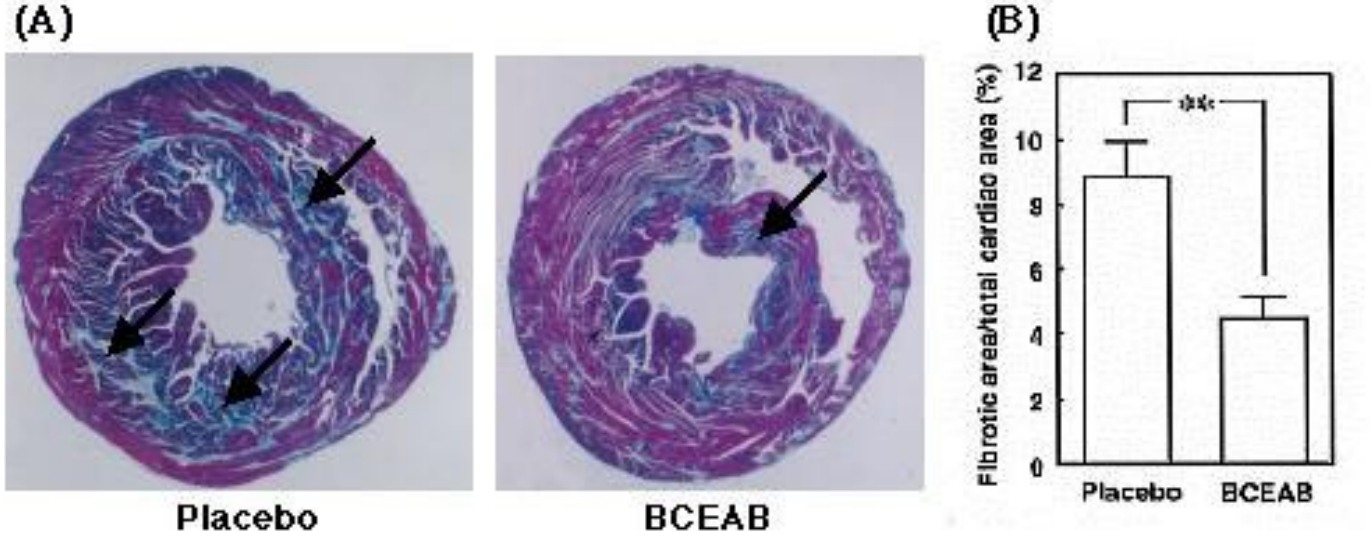
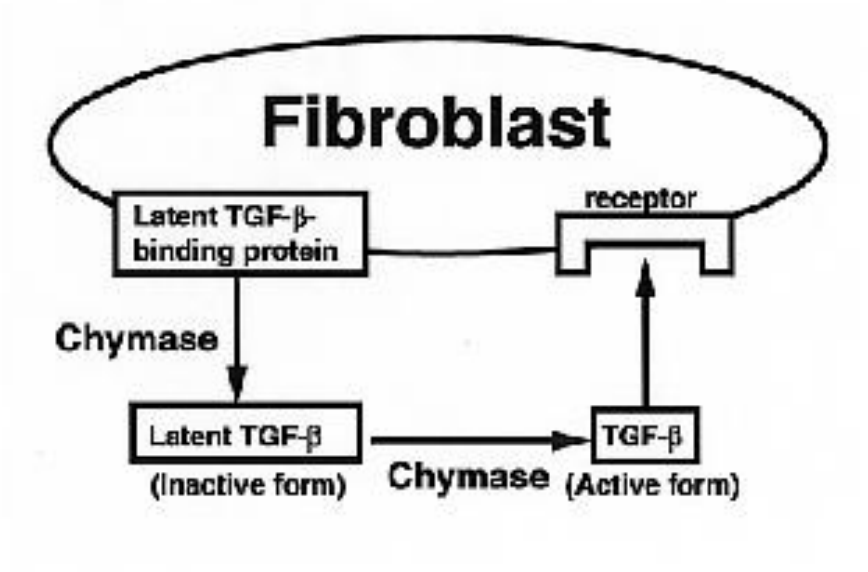

(A)
Placebo
BCEAB
(B)
Fibrotic area/total cardiac area (%)
12
10
8
6
4
2
0
**
Placebo
BCEAB

Fig. 8


Fibroblast
Latent TGF-β-
binding protein
receptor
Chymase
Latent TGF-β
(Inactive form)
Chymase
TGF-β
(Active form)