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Effect of Angiotensin II Type 1 Receptor Blocker on Cardiac Angiotensin-Converting Enzyme and Chymase-Like Activities, and Cardiac Fibrosis in Cardiomyopathic Hamsters

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ABSTRACT. It has been reported that cardiac chymase has an effect on cardiac fibrosis through the Angiotensin (Ang) II formation and an Ang II-independent mechanism. In the present study, Ang II type 1 (AT1) receptor blocker (candesartan cilexetil) was administered to dilated cardiomyopathic (DCM; Bio TO2) hamsters for 4 weeks to study the effect of AT1 receptor blocker on cardiac chymase-like activity and cardiac fibrosis. Echocardiography, histological examination, and assessment of cardiac angiotensin-converting enzyme (ACE)/chymase-like activities were conducted. Hamsters showed cardiac dysfunction due to increased left ventricular dimensions and decreased ventricular wall thickness, significant increase in cardiac chymase-like activity, and fibrosis. This result indicates that the cardiac chymase-like activity is responsible for cardiac fibrosis. When candesartan cilexetil was administered to Bio TO2 hamsters, cardiac chymase-like activity increased significantly, whereas cardiac fibrosis decreased significantly. Cardiac ACE and chymase-like activities were unchanged in non-DCM hamsters with candesartan cilexetil. This suggests that the cardiac Ang II formation mechanism was stimulated by suppressing the effect of cardiac Ang II, and cardiac chymase-like activity could be increased. Moreover, this mechanism may be more highly activated if cardiac Ang II is activated in the heart. In conclusion, we demonstrated that AT1 receptor blocker reduced cardiac fibrosis, although cardiac chymase-like activity increased. Because the Ang II-forming pathway and the effect of chymase in hamsters is similar to that in dogs, the results of the present study may supplement the available information for dogs.

KEY WORDS: angiotensin-converting enzyme, chymase, heart failure, remodeling, renin-angiotensin system.

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The renin-angiotensin system (RAS) is an adaptive mechanism of the body that maintains circulatory homeostasis and cardiac function in the presence of excessive hemodynamic overload. Although it has been thought that RAS exists only in the circulatory system (circulating RAS), RAS also has been found to exist in the heart (cardiac RAS) [14, 24]. Angiotensin (Ang) II, one of the effector peptides of RAS, is known as a causative agent of cardiac remodeling [15]. The Ang II-forming mechanism in the dog's heart is the same as that in humans. In both dogs and humans, cardiac Ang II is produced not only by the cardiac angiotensin-converting enzyme (ACE)-dependent pathway, but also by the cardiac chymase-dependent pathway [1, 2, 23]. The presence of this alternative pathway indicates that ACE inhibitors cannot completely inhibit the cardiac Ang II. A study in rats revealed that Ang II type 1 (AT1) receptor blockers reduced the hemodynamic load and cardiac remodeling by inhibiting the effect of Ang II at the receptor level, and consequently improved cardiac function [20]. Due to this difference in the effector site, AT1 receptor blockers are expected to be more superior to ACE inhibitors. However, previous reports in dogs with heart failure demonstrated that AT1 receptor blockers did not improve cardiac fibrosis [13, 21]. Differences between rats and dogs can be caused by the cardiac Ang II-forming mechanism and the effect of cardiac chymase. In the rat heart, Ang II is mainly produced by cardiac ACE, not by cardiac chymase, and is degraded by cardiac chymase [16].

Because of the difference in cardiac Ang II-forming pathways by species, hamsters were used in this study to examine the effect of AT1 receptor blockers on cardiac fibrosis in dogs. After 4 weeks of administration of an AT1 receptor blocker to the hamsters, echocardiography, assessment of cardiac Ang II-forming enzymes, and histological examination of the heart were conducted to investigate the relationships between cardiac RAS and cardiac function/remodeling.

MATERIAL AND METHODS

Animals: Twelve 16 week old male dilated cardiomyopathic (DCM) hamsters (Bio TO2 hamster, Bio Breeders Inc., Fitchburg, MA, U.S.A.) were used as genetic models of heart failure. In addition, 14 age-matched male non-myopathic control (non-DCM) hamsters (Bio F1B hamster, Bio Breeders Inc., Fitchburg, MA, U.S.A.) were used as healthy controls. For investigation of the cardioprotective effect of an AT1 receptor blocker, candesartan cilexetil, 16–20 week old DCM and non-DCM hamsters were used. DCM and non-DCM hamsters were randomly distributed into two groups, with each group consisting of equal numbers of both DCM and non-DCM hamsters. The first group (DCM-ARB

and non-DCM-ARB) was treated with candesartan cilexetil (10 mg/kg/day). The second group (DCM-Placebo and non-DCM-Placebo) was treated with a placebo (5% arabic gum solution) and was used as an untreated cardiomyopathic control. The dosage of candesartan cilexetil was based on the report of Nakamura *et al.* [10]. Our pilot study (data is not shown) also indicated that the dosage was adequate for hamsters. Drugs were administered daily by gavage. All animals were individually housed under climate-controlled conditions with a 12-hr light/dark cycle and were provided with standard food (lab MR stock, Nihon Nosan Kogyo K.K., Kanagawa, Japan) and tap water *ad libitum*. The study conformed to *The Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

The body weight of the hamsters was measured biweekly. Hamsters underwent echocardiography at 16 (before treatment) and at 20 weeks of age (4 weeks after the treatment), and were sacrificed for cardiac morphometric and biochemical examinations at 20 weeks of age.

Serial echocardiographic assesment: Echocardiographic measurements were obtained from all hamsters in each group at 16 and 20 weeks of age. The chest of each hamster was shaved, and the animal was positioned in left lateral recumbency under anesthesia using intraperitoneal administration of ketamine hydrochloride (60 mg/kg) and xylazine (5 mg/kg). Echocardiography was performed using an ultrasound (ProSound SSD-5000, ALOKA Co., Ltd, Japan) equipped with a 10.0-MHz phase-array transducer (UST-5296, ALOKA Co., Ltd, Japan). M-mode images were obtained from an optimized two-dimensional short-axis view of the left ventricle. Interventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), and left ventricular internal diameter in the diastolic and systolic phases (LVIDd and LVIDs, respectively) were measured on three consecutive cycles by a single observer. Left ventricular fractional shortening (FS) was calculated using the following formula: (LVIDd-LVIDs)/LVIDd × 100. The mean left ventricular wall thickness (LVWT) was defined as the average of IVS and LVPW. The ratio of LVID to LVWT (LVID/LVWT) was calculated for assessment of the left ventricular remodeling process.

Heart collection: Anesthesia was induced in each hamster using intraperitoneal administration of pentobarbital (50 mg/kg). Median sternotomy was performed, and the heart was exposed. Potassium chloride (20 mmol/kg) was then injected into the left ventricle to achieve a cardiac arrest in diastole. The heart was excised, and the blood was washed out with ice-cold saline. After the atrium was cut off, the right and left ventricles were weighed and the ventricular weight-to-body weight ratio calculated. The heart was cut cross-sectionally at the mid-papillary muscle level, and the apex side of the heart was frozen in liquid nitrogen and stored in a deep freezer at –80°C until measurement of ACE and chymase-like activities. The remaining hearts from each group were fixed in 10% formaldehyde and subjected to histological examination.

Histological examination: The ventricles were embedded in paraffin and cut into thin sections (3 μm) using standard procedures. They were then stained with hematoxylin-eosin and picrosirius red stain. The collagen density percentage was determined for 4 regions, the subendocardial, subepicardial, and middle portion of the left ventricle and right ventricle. Each region was assessed with picrosirius red staining in 10 random fields with a magnification of × 200. The collagen density percentage was calculated using a computerized morphometry system (Mac SCOPE Ver 2.69.1, Mitani Co., Fukui, Japan), and the sum of all the areas stained positive for sirius red was divided by the sum of all myocardial areas for each hamster.

Measurement of cardiac ACE and chymase-like activities: Cardiac ACE and chymase-like activities were measured as described in a previous report [12]. One unit of ACE activity was defined as the amount of enzyme that produced 1 μmol of hippuric acid from Hippuryl-His-Leu per minute. Cardiac chymase-like activity was defined by chymostatin-inhibitable Ang II formation and was expressed as the amount of Ang II formed per mg of protein per minute.

Drugs: The following drugs were obtained from Sigma (St. Louis, MO, U.S.A.): angiotensin II acetate salt, aprotinin, o-phenanthroline, chymostatin, captopril, and hippuric acid hydrate. Angiotensin I was obtained from Bachem (U.S.A.). Hippuryl-His-Leu was obtained from Peptide Institute (Japan). Five percent arabic gum was obtained from Wako Pure Chemical Industries, Ltd. (Japan). Candesartan cilexetil was obtained from Takeda Pharmaceutical Company Limited (Japan).

Statistical analysis: All data are presented as means ± standard error (SEM). Between-group comparisons and within-group comparisons at each time period were performed using a two-tailed Student's *t*-test. The level of significance was set at $p<0.05$.

RESULTS

Clinical findings: During the experiment, no hamsters were removed due to death, and none showed signs of such things as pneumonedema and ascites.

Body weight and ventricular weight: The detailed data are summarized in Table 1. The DCM-Placebo group showed a significantly lower body weight (g) at 16 and 20 weeks of age ($p<0.01$) and a significantly lower ventricular weight (mg) ($p<0.01$) than the non-DCM-Placebo group. The DCM-ARB group had significantly higher ventricular weight than the DCM-Placebo group ($p<0.05$). However, there were no statistical differences between the DCM-Placebo and non-DCM-Placebo groups, the non-DCM-Placebo and non-DCM-ARB groups, and the DCM-Placebo and DCM-ARB groups in ventricular weight-to-body weight ratio.

Echocardiography: The detailed data are summarized in Table 2. At 16 weeks of age, an increase in LVIDd ($p<0.01$) and a decrease in IVSd ($p<0.01$) and LVPWd ($p<0.01$) was observed in the DCM-Placebo group compared with the

Table 1. Body weight, ventricular weight, and ventricular weight-to-body weight ratio

	16w BW (g)		20w BW (g)		VW (mg)		VW/BW (mg/g)	
	Placebo	ARB	Placebo	ARB	Placebo	ARB	Placebo	ARB
Non-DCM	120.9 ± 2.9	121.2 ± 2.8	124.9 ± 2.5	128.0 ± 4.0	335.1 ± 5.1	345.0 ± 8.5	2.69 ± 0.06	2.70 ± 0.05
DCM	102.4 ± 1.8£	106.7 ± 2.5	105.6 ± 2.7£	113.9 ± 2.9	270.4 ± 7.9£	294.4 ± 5.2†	2.56 ± 0.05	2.59 ± 0.06

n=6–7. All data are presented as means ± standard error (SEM). BW: body weight; VW: ventricular weight; VW/BW: ventricular weight-to-body weight ratio. Non-DCM: Bio F1B; DCM: Bio TO2; Placebo: treated with placebo; ARB: treated with candesartan cilexetil, 10 mg/kg/day. £ $p<0.01$ versus the age-matched non-DCM-Placebo group; † $p<0.05$ versus the DCM-Placebo group.

Table 2. Echocardiographic data at 16 and 20 weeks of age

	IVSd (mm)		LVPWd (mm)		LVIDd (mm)		LVIDd / LVWTd		FS (%)	
	Placebo	ARB	Placebo	ARB	Placebo	ARB	Placebo	ARB	Placebo	ARB
16 weeks										
Non-DCM	0.176 ± 0.009	0.168 ± 0.005	0.187 ± 0.009	0.181 ± 0.003	0.358 ± 0.014	0.357 ± 0.012	2.015 ± 0.142	2.082 ± 0.092	45.1 ± 3.5	46.8 ± 2.6
DCM	0.138 ± 0.005£	0.132 ± 0.010	0.144 ± 0.006£	0.135 ± 0.010	0.445 ± 0.017£	0.433 ± 0.026	3.225 ± 0.195£	3.738 ± 0.597	26.9 ± 2.1£	29.8 ± 3.2
20 weeks										
Non-DCM	0.190 ± 0.007	0.197 ± 0.005¶	0.198 ± 0.010	0.215 ± 0.003¶	0.379 ± 0.011	0.350 ± 0.020	2.001 ± 0.101	1.725 ± 0.091ç	41.8 ± 2.6	41.9 ± 2.9
DCM	0.117 ± 0.003£¶	0.110 ± 0.000	0.117 ± 0.003£¶	0.124 ± 0.004	0.464 ± 0.018£	0.497 ± 0.014	4.004 ± 0.175£ç	4.288 ± 0.148	25.3 ± 1.9£	22.2 ± 1.7

n=6–7. All data are presented as means ± standard error (SEM). IVSd: diastolic interventricular septal thickness; LVPWd: diastolic left ventricular posterior wall thickness; LVIDd: left ventricular end-diastolic internal diameter; LVIDd/LVWTd: ratio of LVIDd to the mean diastolic left ventricular wall thickness (LVWT); FS: left ventricular fractional shortening. 16 weeks: studied at 16 weeks of age before examination; 20 weeks: studied at 20 weeks of age, at the end of examination. Non-DCM: Bio F1B; DCM: Bio TO2; Placebo: treated with placebo; ARB: treated with candesartan cilexeetil, 10 mg/kg/day. £ $p<0.01$ versus the age-matched non-DCM-Placebo group; ¶ $p<0.01$ versus 16 weeks of age in the same group; ç $p<0.05$ versus 16 weeks of age in the same group.

non-DCM-Placebo group. In the DCM-Placebo group, enlargement of the heart chamber and a decrease in the wall thickness resulted in a significant increase in LVID/LVWT in the diastolic phases (LVIDd/LVWTd) ($p<0.01$) and a decline in FS ($p<0.01$) compared with the non-DCM-Placebo group. There were no statistically significant differences in LVIDd/LVWTd and FS between the non-DCM-Placebo and non-DCM-ARB groups, and the DCM-Placebo and DCM-ARB groups.

In the DCM-Placebo group, IVSd ($p<0.01$) and LVPWd ($p<0.01$) decreased significantly, and LVIDd/LVWTd ($p<0.05$) increased significantly between 16 and 20 weeks of age. However, LVIDd and FS showed no significant differences between 16 and 20 weeks of age. The decrease in IVSd and LVPWd between 16 and 20 weeks of age can be attributed to the effect of DCM because this decrease was not observed in the non-DCM-Placebo group.

Administration of candesartan cilexetil to the non-DCM group did not cause significant differences in LVIDd and FS between 16 and 20 weeks of age. However, IVSd ($p<0.01$) and LVPWd ($p<0.01$) increased significantly, and LVIDd/LVWTd ($p<0.05$) decreased significantly between 16 and 20 weeks of age.

Administration of candesartan cilexetil to the DCM group did not elicit significant inhibition of the increase of LVIDd or significant suppression of the decrease in IVSd, LVPWd, and FS compared to the DCM-Placebo group. However, there were no significant decreases between 16 and 20 weeks of age in IVSd, LVPWd, and FS, and no significant increases between 16 and 20 weeks of age in LVIDd and LVIDd/LVWTd in the DCM-ARB group.

Left ventricular histological examination: Histological features of the subendocardial region of the left ventricle obtained at 20 weeks of age in the DCM and non-DCM groups are shown in Fig. 1. The detailed data are summarized in Table 3. The DCM groups showed a significant increase in interstitial cardiac fibrosis.

Administration of candesartan cilexetil to the non-DCM hamsters did not cause significant differences in cardiac fibrosis in the subendocardial and subepicardial regions or middle portion of the left ventricle and right ventricle.

Administration of candesartan cilexetil to the DCM hamsters produced lower percentages of fibrosis of the subepicardial regions of the left ventricle ($p<0.05$) and right ventricle ($p<0.05$) compared with the DCM-Placebo group, although there were no significant differences in the percentages of fibrosis of the subendocardial and middle portion of the left ventricle between the DCM-Placebo and DCM-ARB groups.

ACE and chymase-like activities in the heart: Cardiac ACE and chymase-like activities of the DCM and non-DCM groups are shown in Fig. 2. There were no differences in the

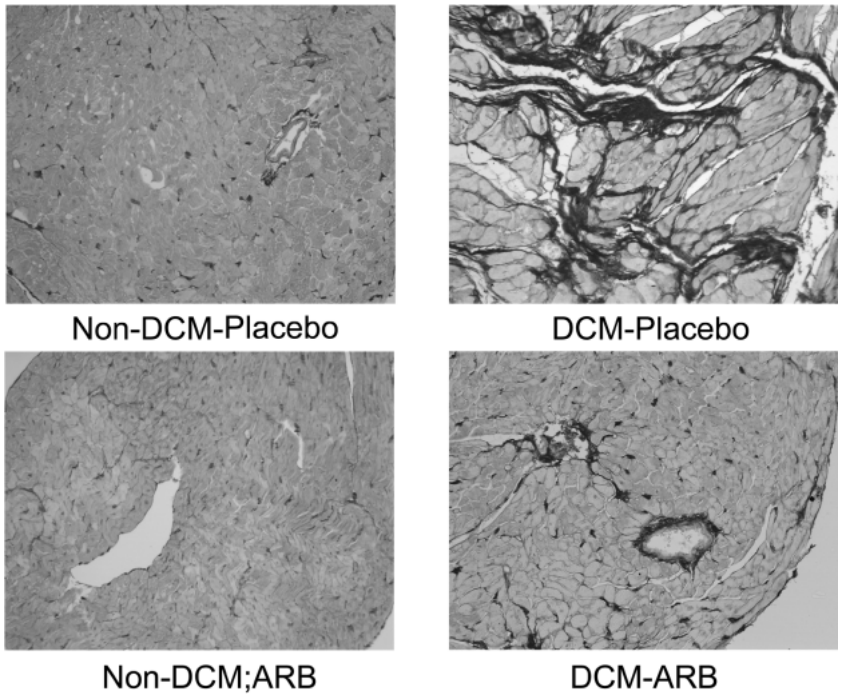

Fig. 1. Histological appearance of the subendocardial region of the left ventricle obtained at 20 weeks of age from the DCM and non-DCM groups (original magnification of × 200). The collagen was stained in high density. Non-DCM-Placebo: Bio F1B treated with placebo; Non-DCM-ARB: Bio F1B treated with candesartan cilexetil, 10 mg/kg/day; DCM-Placebo: Bio TO2 treated with placebo; DCM-ARB: Bio F1B treated with candesartan cilexetil, 10 mg/kg/day.

Table 3. Collagen density percent at 20 weeks of age

	Left ventricle						Right ventricle	
	Subendocardial		Subepicardial		Middle			
	Placebo	ARB	Placebo	ARB	Placebo	ARB	Placebo	ARB
Non-DCM	2.081	1.736	2.250	2.653	1.868	1.753	3.116	2.445
	± 0.106	± 0.173	± 0.214	± 0.150	± 0.219	± 0.095	± 0.307	± 0.170
DCM	6.369	4.341	9.736	6.013	10.206	8.423	9.049	6.493
	± 0.620£	± 0.910	± 1.018£	± 0.228†	± 1.533£	± 0.729	± 0.670£	± 0.893†

n=6–7. All data are presented as means ± standard error (SEM). Non-DCM: Bio F1B; DCM: Bio TO2; Placebo: treated with placebo; ARB: treated with candesartan cilexetil, 10 mg/kg/day. £ $p<0.01$ versus the non-DCM-Placebo group; † $p<0.05$ versus the DCM-Placebo group.

cardiac ACE activity between the DCM-Placebo and non-DCM-Placebo groups (0.17 ± 0.03 and 0.12 ± 0.02 mU/mg protein, respectively). Administration of candesartan cilexetil in the non-DCM group did not cause a significant difference in cardiac ACE activity (0.12 ± 0.02 mU/mg protein) compared with the non-DCM-Placebo group (0.12 ± 0.02 mU/mg protein). Similarly, administration of candesartan cilexetil in the DCM group did not cause a significant difference in the cardiac ACE activity (0.29 ± 0.06 mU/mg protein) compared with the DCM-Placebo group (0.17 ± 0.03 mU/mg protein).

Cardiac chymase-like activity increased significantly in the DCM-Placebo group compared with the non-DCM-Placebo group (0.76 ± 0.31 and 0.14 ± 0.06 nmol/min/mg protein, respectively, $p<0.05$). Administration of candesartan cilexetil in the non-DCM group did not produce a significant difference in cardiac chymase-like activity (0.13 ± 0.08 nmol/min/mg protein) compared with the non-DCM-Placebo group (0.14 ± 0.06 nmol/min/mg protein). However, administration of candesartan cilexetil in the DCM group produced greater cardiac chymase-like activity (1.50 ± 0.15 nmol/min/mg protein, $p<0.05$) compared with the DCM-Placebo group (0.76 ± 0.31 nmol/min/mg protein).

DISCUSSION

Cardiac chymase has an effect on the pathophysiology of heart failure through the production of Ang II [7, 8]. Ham-

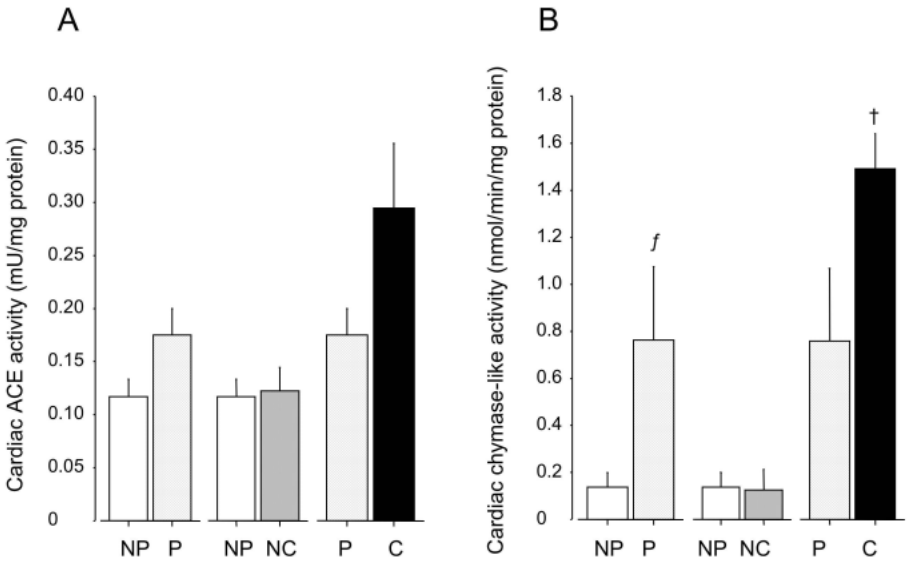

Fig. 2. Cardiac angiotensin-converting enzyme (ACE: A) and chymase-like activity (B) in the DCM and non-DCM groups. NP: Bio F1B treated with placebo (non-DCM-Placebo); NC: Bio F1B treated with candesartan cilexetil, 10 mg/kg/day (non-DCM-ARB); P: Bio TO2 treated with placebo (DCM-Placebo); C: Bio TO2 treated with candesartan cilexetil, 10 mg/kg/day (DCM-ARB). *f* $p<0.01$ versus the non-DCM-Placebo group; † $p<0.05$ versus the DCM-Placebo group.

sters are good model animals to study the pathophysiology of the heart and the effect of AT1 receptor blockers in dogs with cardiac disease because they have an alternative Ang II-forming pathway in the heart [11], and Ang II leads to cardiac fibrosis as in dogs. The mechanism of cardiac Ang II on cardiac fibrosis in hamsters may be considered similar to that in dogs. Using the hamster as an experimental animal instead of dogs is important for the purpose of animal protection. The lifespan of hamsters is shorter than that of dogs, and use of hamsters enables researchers to evaluate the effect of AT1 receptor blockers more easily and in a shorter period of time. In fact, Bio TO2 hamsters show signs of severe congestive heart failure at 11 months of age [3]. In the present study, Bio TO2 hamsters showed a significant increase in interstitial cardiac fibrosis at 20 weeks of age.

The present study investigated cardiac ACE and chymase-like activities and evaluated the effect of AT1 receptor blocker for cardiac fibrosis. It has been reported that cardiac ACE activity in Bio TO2 hamsters was increased significantly in the advanced heart failure phase, but was not increased at 8 weeks of age, the beginning of the cardiac remodeling phase [3]. Other studies using hypertrophic cardiomyopathic hamsters have shown that cardiac chymase activity increased significantly in both the hypertrophic and fibrotic phases, and that cardiac chymase contributed to the progress of cardiomyopathy through the production of cardiac Ang II [17]. However, there are no reports on cardiac chymase activity in dilated cardiomyopathic hamsters. Bio TO2 hamsters at 20 weeks of age showed a significant increase in cardiac chymase-like activity and cardiac fibrosis without showing an increase on cardiac ACE activity. This result indicates that this phase is the cardiac remodeling stage and that the cardiac chymase-like activity is responsible for pathological changes, such as cardiac fibrosis through production of Ang II. This indicates that the present hamsters are appropriate model animals presenting the Ang II-induced cardiac fibrosis, and it is expected that AT1 receptor blocker inhibits cardiac fibrosis.

Increased cardiac chymase-like activity in Bio TO2 hamsters may lead to activation of cardiac Ang II. When AT1 receptor blocker was administered to the Bio TO2 hamsters, cardiac chymase-like activity increased significantly, while in Bio F1B (non-DCM) hamsters, both cardiac ACE and chymase-like activities were unchanged. A previous report in rat cultured ventricular fibroblasts revealed that angiotensinogen production by cardiac fibroblasts is under negative feedback control of Ang II [5]. Although regulation of Ang II by AT1 receptor blocker was not studied in the present study, it is suggested that the Ang II-forming mechanism is stimulated if the effect of cardiac Ang II is blocked and cardiac Ang II-forming enzyme (chymase) is increased. Moreover, this mechanism may be activated more strongly if cardiac Ang II is activated in the heart.

AT1 receptor blockers have been reported to improve the cardiac structure and function by way of reducing the hemodynamic load [9, 22], but no improvement was observed in the present study. A possible reason for this result was considered to be that the Bio TO2 hamsters revealed an inherited pathophysiological change, such as a decrease in wall

thickness due to enlargement of the heart chamber, during the 4 weeks and the AT1 receptor blocker could not improve the inherited changes. Because cardiac chymase is responsible for cardiac fibrosis in DCM hamsters [17], AT1 receptor blocker was expected to improve the cardiac fibrosis. In fact, AT1 receptor blocker reduced the cardiac fibrosis, although cardiac chymase-like activity was increased. It has been reported that cardiac chymase might play an important role in the progression of cardiac fibrosis via activation of transforming growth factor-β [6, 18, 19]. In the present study, however, cardiac fibrosis was reduced because AT1 receptor blocker works as an inhibitor of cardiac Ang II in spite of increasing cardiac chymase-like activity.

The Bio TO2 hamsters in the present study allowed the researchers to study the effect of AT1 receptor blocker. It has been reported that cardic ACE and chymase-like activity, especially chymase-like activity, increased in the cardiac tissue of dogs with heart failure [4]. Considering the similarities between hamsters and dogs in the Ang II-forming pathway and the effect of chymase, the results in hamster may supplement the available information for dogs with heart failure. The availability for treatment of AT1 receptor blocker in dogs is not yet fully understood, and we hope that it will be demonstrated with this experimental model.

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