Matrix Metalloproteinase Activity in Rats

Doxycycline Attenuates Isoproterenol-Induced Myocardial Fibrosis and Matrix Metalloproteinase Activity in Rats

Doxycycline attenuates isoproterenol-induced myocardial fibrosis and matrix metalloproteinase activity in rats. Twenty-four Wistar-Kyoto rats were divided into 3 groups: control (CTL; n=8), isoproterenol (ISO; n=8), and isoproterenol with doxycycline (ISO+DOX; n=8). ISO and ISO+DOX rats received i.-isoproterenol (2.0 mg/kg/d) for 14 d, whereas the CTL group received vehicle. In addition, ISO+DOX rats received a subcutaneous injection of doxycycline (25 mg/kg/d) for 14 d, whereas CTL and ISO rats were injected with saline. Cardiac fibrosis was evaluated via histopathological analysis. MMP-2 and -9 were analyzed by Western blotting and zymography. Compared to the control, the myocardial cross-sectional area and areas of fibrosis were increased significantly in the ISO group, but were attenuated in the ISO+DOX group. MMP-2 activity also increased significantly in the ISO group, but decreased in the ISO+DOX group. Similarly, immunoblotting showed significant increase in MMP-2 and -9 levels in the ISO group, and decreased levels in the ISO+DOX group. Our results suggest that the enhanced expression of MMPs plays a prominent role in promoting myocardial fibrosis in β-agonist signaling pathway, and that MMP-inhibiting compounds may attenuate myocardial fibrosis.

Key words matrix metalloproteinase; doxycycline; fibrosis; isoproterenol

MATERIALS AND METHODS

Four-week-old Wistar-Kyoto rats (n=42) were obtained from a commercial laboratory (Charles River, Yokohama, Japan). The rats were housed individually in an air-conditioned room with a 12-h dark–light cycle and were given a standard diet with ad libitum access to tap water. The rats were divided into 3 groups: control (CTL), isoproterenol (ISO), and isoproterenol with doxycycline (ISO+DOX). This study was approved by the Institutional Laboratory Animal Care and Use Committee of the School of Veterinary Medicine of Kitasato University, Japan.

Study 1 Four-week-old rats (n=18; body weight, 90—110 g) were divided into 3 groups: control (n=6), ISO (n=6), and ISO+DOX (n=6). Animals were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). A 3Fr catheter was implanted into the jugular vein and the drug was administered with an infusion pump (Nihon Kohden, Tokyo, Japan). The rats were stabilized for 15 min and placed in the prone position above an ultrasound standoff pad (3M Health Care Ltd., Tokyo). Control rats were infused 0.9% saline alone. i.-isoproterenol (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) was infused at 0.5 and 1.0 μg/kg/min for 5 min. In ISO+DOX rats, doxycycline hydrochloride was infused at 17—18 μg/kg/min (25 mg/kg/d; Sigma-Aldrich Co.) concomitant with i.-isoproterenol.

Echocardiographic Measurements The heart rate was monitored using an echocardiographic system. As an index of left ventricular contractility, fractional shortening (FS) was measured by echocardiography using a 12-MHz probe (Philips Electronics Japan, Tokyo, Japan). M-mode measurements of systolic and end-diastolic left ventricular internal diameter (LVIDs and LVIDd) were measured from the short axis view. Left ventricular FS was calculated as follows:
The average of three cardiac cycles was calculated. Data were stored digitally and analyzed off-line by a single observer. All measurements were performed before and after administering the isoproterenol.

**Study 2** Seven-week-old rats (*n*=24; body weight, 200—280 g) were divided into 3 groups: CTL (*n*=8), ISO (*n*=8), ISO+DOX (*n*=8). After being anesthetized, rats were aseptically implanted with a subcutaneous osmotic minipump (model 2002, Alzet, Durect Co., U.S.A.) through a small intercostal incision. These minipumps were used to deliver 2.0 mg/kg/d of l-isoproterenol dissolved in a 0.9% saline for 14 d. Control rats were implanted with minipumps containing vehicle (0.9% saline) alone. During the treatment period, rats were also given a subcutaneous injection of doxycline hydrochloride (ISO+DOX rats; 25 mg/kg/d, 0.5 ml *bis in die* (BID)) or saline (CTL and ISO rats; 0.5 ml, BID).

**Tissue Sampling** After 14 d of treatment, the hearts were excised with the use of sodium pentobarbital (50 mg/kg, intraperitoneally (i.p.)) anesthesia. The heart was divided into the right ventricle (RV) and LV including the septum, in ice-cold saline. The whole heart weight (HW) and the LV weight (LVW) were measured, corrected for body weight, and used to determine the HW/BW and LVW/BW ratios. The hearts were cut into coronal sections at the level of the papillary muscles; the basal part of the heart was fixed in 10% formalin, and the apex of the heart was snap-frozen in liquid nitrogen.

**Histopathological Analysis** Fixed hearts were embedded in paraffin and transverse sections (4 μm) were cut and stained by routine methods. Azan stain was used to detect collagen. To determine the degree of cardiac fibrosis, images of Azan-stained samples were transferred into a computer using a microscope. A minimum of 10 fields from each LV section were scored at 100× magnification. The degree of cardiac fibrosis was determined based on the area of fibrosis divided by the total area (%cardiac fibrosis). A minimum of 100 cells from each LV section at 400× magnification were quantified as described above. Marker was used as a control (Gelatin Zymo MMP Marker, AlphaEase FC, Alpha InnoTech Co., CA, U.S.A.).

**Western Blotting** Western blotting analyses for MMP-2 and -9 were performed according to a modification of a method described previously. Frozen tissue was homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA2Na, 0.5% Triton X-100], centrifuged at 12000×*g* for 15 min, and the resultant supernatant was used for analysis. Protein content was determined using the Bradford technique. Tissue lysates were diluted in sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 0.04% bromphenol blue] and aliquots with a final protein content of 5 μg were separated by 10% SDS-polyacylamide electrophoresis (SDS-PAGE) on gels containing 0.6% gelatin. After SDS-PAGE, the gels were washed twice in extraction buffer [20 mM Tris–HCl (pH 7.5), 2 mM CaCl₂, 1 μM ZnCl₂, 0.02% NaN₃, 2.5% Triton X-100] for 30-min each time and rinsed in water. Gels were incubated at 37°C overnight in incubation buffer [20 mM Tris–HCl (pH 7.5), 2 mM CaCl₂, 1 μM ZnCl₂, stained with Coomassie staining solution (0.5% Coomassie R250, 30% methanol, 10% acetic acid) for 2 h, and then de-stained in distilled deionized H₂O. The MMP marker was used as a control (Gelatin Zymo MMP Marker, Life Laboratory Co., Yamanaka, Japan). Gelatinolytic bands were quantified as described above.

**Statistical Analysis** All numerical data are expressed as mean±S.D. Changes in echocardiographic measurements, heart rate and FS were compared to baseline using a one-factor repeated measures analysis of variance (ANOVA). These measurements from baseline were compared to the control group using two-way ANOVA. Histopathological analysis, Western blotting, and MMP zymography were compared using one-way ANOVA. The significances of differences between the mean values at baseline and under each condition were tested using Tukey’s multiple comparison test. The differences were considered significant at a *p*-value of <0.05.

**RESULTS**

**Study 1** Isoproterenol administration significantly increased the heart rate from baseline in ISO and ISO+DOX groups (*p*<0.05 for both; Fig. 1A). Similarly, isoproterenol administration significantly increased the FS from baseline in ISO (*p*<0.01 and *p*<0.001, respectively) and ISO+DOX groups (*p*<0.05 and *p*<0.01, respectively; Fig. 1B). These responses were insignificant between ISO and ISO+DOX groups, but were significantly elevated compared to those in the CTL group (HR; *p*<0.05 for both, FS; *p*<0.001 for both).

**Study 2** The HW/BW and LVW/BW ratios increased significantly in the ISO and ISO+DOX groups compared to the control (Table 1), but did not differ between the ISO and...
ISO/H11001 DOX groups. Compared with the controls, the MCSA were significantly increased by ISO treatment. Compared to ISO treatment, ISO/H11001 DOX treatment significantly decreased the MCSA. Pathological findings for each group are shown in Fig. 2. The percent fibrotic area increased significantly in the ISO and ISO/H11001 DOX groups compared to the control (p<0.001 for both), but decreased significantly in the ISO+DOX group compared to the ISO group (p<0.05).

ProMMP-2 activity increased significantly in the ISO group compared to the control (p<0.001), but decreased significantly in the ISO+DOX group compared to the ISO group (p<0.01, Fig. 3). ProMMP-2 activity did not differ between the ISO+DOX and CTL groups. No MMP-9 activity was detected in heart tissue via zymography. Immunoblotting revealed significant increases in MMP-2 and -9 protein expression in the ISO group compared to the control (p<0.01 for both), but significant decreases were observed in the ISO+DOX group compared to the ISO group (p<0.01 and p<0.05, respectively; Figs. 4 and 5). MMP-2 and -9 levels did not differ significantly between the ISO+DOX and CTL groups.

DISCUSSION

Isoproterenol is a well-known β-adrenoceptor agonist that produces inotropic and chronotropic effects in a dose-dependent manner.18—20) Our findings were consistent with previous studies, which have shown that isoproterenol induces an increase in heart rate and FS. In animals, isoproterenol has been shown to enhance LV cardiac systolic and diastolic function and heart rate.19—21) Therefore, the cardiac responses to isoproterenol observed in the present study represented typical reactions. Although doxycycline is a broad spectrum antibiotic with MMP inhibition, it is not clear yet whether or not it directly inhibits β-adrenoceptor. In the present study, doxycycline did not affect the cardiac responses to
thetatic nervous system or the renin–angiotensin–aldosterone system is the predominant cause of myocardial fibrosis in patients with heart disease. In addition, collagen accumulation impairs cardiac function, which is associated with the development of heart failure. Recent clinical studies have reported increased MMP expression and activation in myocardial fibrosis associated with several heart diseases, including aortic stenosis, hypertrophic cardiomyopathy, and dilated cardiomyopathy. Patients with hypertrophic cardiomyopathy had higher levels of procollagen type III amino-terminal propeptide and collagen I carboxy-terminal telopeptide than normal subjects, concomitant with MMP-2 and -9 expression. In patients with aortic stenosis, the expression/activation of MMPs and collagen accumulation increased in proportion to disease severity. Similarly, myocardial fibrosis was aggravated in animal models of heart disease, in association with the expression and activation of MMPs. Transgenic mice over-expressing MMP-1 showed marked deterioration of cardiac function. In addition, previous studies showed that isoproterenol causes myocardial hypertrophy and apoptosis through the augmentation of mitogen-activated protein kinase (MAPK) expression, which related to the expression/activation of MMPs. In the present study, 2-week administration of isoproterenol induced cardiac hypertrophy and myocardial fibrosis and increased MMP-2 and -9 protein expression. Our results are consistent with those of previous reports indicating that β-agonists augment myocardial apoptosis, hypertrophy, and MMP activation in rats. These observations suggest that MMP synthesis/activation is enhanced by the isoproterenol administration and is associated with myocardial fibrosis.

MMPs are known to play a significant role in collagen turnover in pathophysiological processes. Selective MMPi treatment inhibited MMP activity and subsequently preserved cardiac function and attenuated LV enlargement in a porcine heart disease model. In other studies, a selective MMPi prevented collagen accumulation and MMP activation and preserved cardiac function in animal models of chronic heart failure. In addition, MMP-9 knockout mice and rats reported increased MMP expression and activation in myocardial fibrosis. Furthermore, the survival rate was significantly higher in MMP-2 knockout mice with myocardial infarction than in control mice.

Doxycycline is a common tetracycline antibiotic, and is a broad-spectrum inhibitor of MMP expression and activity. Previous studies showed that pre-treatment with doxycycline reduced infarct size in rats and that early short-term treatment with doxycycline after myocardial infarction preserved left ventricular structure (i.e., heart weight, myocyte size, and internal LV diameter). Similarly, Errami et al. reported that although isoproterenol causes cardiac hypertrophy and MMPs expression/activation through the augmentation of MAPK expression in mice, doxycycline attenuated these effects. In vitro, doxycycline prevented myocardial apoptosis, as evidenced by the frequency of TUNEL-positive cells, and decreased MMP-2 and caspase-3 activity in myocytes treated with tumor necrosis factor (TNF-α). In the present study, doxycycline treatment attenuated MMP expression/activity, resulting in decreased fibrosis in the rat myocardium.
during chronic isoproterenol administration. Although doxycycline significantly decreased MCSA, it slightly, but not significantly decreased cardiac hypertrophy. Regarding the inconsistency in cardiac hypertrophy with a previous report, this may be explained by methodological differences, i.e., different doses and durations of isoproterenol or different species: Errami et al. administered isoproterenol (40 mg/kg/d) and doxycycline (6 mg/kg/d) for 7 d in mice, but we administered the same drugs for 14 d in rat at, respectively, 2 mg/kg/d and 25 mg/kg/d. These results indicate that the broad-spectrum MMPi, doxycycline, prevents isoproterenol-induced myocardial fibrosis and MMP expression/activity.

**Limitation** We examined the relationship between myocardial fibrosis and MMP expression/activity. Because doxycycline is not a selective inhibitor and can also inhibit other proteases, further studies are required to clarify the mechanisms by which MMPi attenuates myocardial fibrosis. However, the optimal therapeutic dose of doxycycline is yet to be determined and it is possible that treatment with it at different doses and durations may have different results. Other properties of doxycycline, such as the inhibition of protein and collagen synthesis, may have adverse effects on LV remodeling. Finally, doxycycline is the only MMPi currently approved for clinical use, but its application is limited to the treatment of periodontal disease.

**CONCLUSION**

In the present study, chronic administration of the β-agonist isoproterenol induced myocardial fibrosis and was associated with increased MMP expression and activity. Furthermore, doxycycline prevented MMP expression/activity and myocardial fibrosis. These results suggest that enhanced MMP expression plays a prominent role in β-agonist-related myocardial fibrosis, and that MMP-inhibiting compounds may attenuate this condition.

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**REFERENCES**