Effects of Captopril and Telmisartan on Matrix Metalloprotease-2 and -9 Expressions and Development of Left Ventricular Fibrosis Induced by Isoprenaline in Rats

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The aim of this study was to clarify the effects of renin-angiotensin system (RAS) blockade by captopril, an angiotensin converting enzyme inhibitor, and telmisartan, an angiotensin II type 1 receptor antagonist, on matrix metalloproteinase (MMP)-2 and MMP-9 expressions and development of left ventricular (LV) fibrosis induced by isoprenaline in rats. Rats were treated with subcutaneous injection of isoprenaline (5 mg/kg/d) and with oral administration of captopril (30 mg/kg/d) or telmisartan (3 mg/kg/d) for 1 or 7 d. Hearts were excised at the day 2 and day 8. Degree of fibrosis was evaluated by Azan staining. MMP-2 and MMP-9 expressions were analyzed by Western blotting. Localization of MMP-9 expression in LV section was detected by immunohistochemical staining. At the day 8, myocardial fibrosis was observed in LV section from isoprenaline-treated rats. Captopril but not telmisartan significantly enhanced the isoprenaline-induced myocardial fibrosis. MMP-9 expression at the day 2 and MMP-2 expression at the day 8 increased significantly in LV from isoprenaline-treated rats. Captopril had no influence on the MMP-2 expression, but significantly augmented the isoprenaline-induced MMP-9 expression. Telmisartan had no effect on the isoprenaline-induced MMP-2 and MMP-9 expressions. In immunohistochemical staining, MMP-9 positive-interstitial cells were extensively observed in LV sections from isoprenaline + captopril-treated rats at the day 2. The present study reveals that RAS blockade by captopril and telmisartan does not have suppressive effects on isoprenaline-induced MMP-2 and MMP-9 expressions as well as LV fibrosis. Furthermore, captopril may enhance LV fibrosis through promoting isoprenaline-induced MMP-9 expression in cardiac interstitial cells.

Key words matrix metalloproteinase; myocardial fibrosis; isoprenaline; angiotensin converting enzyme inhibitor; angiotensin II type 1 receptor antagonist

Activation of renin-angiotensin system (RAS) and sympathetic nervous system by various cardiovascular diseases, such as myocardial infarction, hypertension, and valvular heart disease, promote cardiac remodeling, including myocardial hypertrophy, myocardial fibrosis, chamber dilatation, and cell death.1,2 Because excessive cardiac remodeling finally induces cardiac dysfunction and heart failure, RAS blockade has been recognised to be beneficial care for heart failure.1,2 Extracellular matrix (ECM) acts as not only a space-filling material but also a bioactive molecule, which modulate cellular adhesion, migration, proliferation, differentiation, and survival.3 Matrix metalloproteinases (MMPs) regulate myocardial ECM turnover and play an important role on cardiac remodeling.4,5 β-Adrenergic stimulation of rats by isoprenaline-treatment has been widely used as a cardiac hypertrophy and fibrosis model.6,7 Several studies have reported that MMP-2 and MMP-9 were upregulated and may play important roles on isoprenaline-induced cardiac remodeling in rats.8—10 We previously demonstrated that RAS blockade by captopril, an angiotensin converting enzyme (ACE) inhibitor, and telmisartan, an angiotensin II type 1 receptor (AT1R) antagonist, attenuated monocrotaline-induced right ventricular (RV) cardiac hypertrophy, fibrosis, dysfunction and MMP-2 and MMP-9 expressions in rats.11,12 Therefore, it is hypothesized that RAS blockade may also suppress MMP-2 and MMP-9 expressions and β-adrenergic stimulation-induced cardiac remodeling. However, little is known about the effects of RAS inhibition on MMPs expressions and development of left ventricular (LV) fibrosis induced by isoprenaline in rats. Thus, we investigated the effects of captopril and telmisartan on MMP-2 and MMP-9 expressions and LV fibrosis induced by isoprenaline in rats.

MATERIALS AND METHODS

Animal Models All animals were treated in accordance with the guidelines for animal treatment of Kitasato University which meet international guiding principles of laboratory animal care. Six-week-old male Wistar rats (Clea Japan Inc., Tokyo, Japan) were maintained on a standard laboratory diet and tap water, and exposed to a 12 h/12 h light–dark cycle at 23 ± 2 °C throughout experiments. Rats were divided into 6 groups: control (CONT), captopril alone- (CAP), telmisartan alone- (TEL), isoprenaline alone- (ISO), isoprenaline+captopril- (ISO+CAP), and isoprenaline+telmisartan (ISO+TEL) treated groups. Isoprenaline bitartrate (5 mg/kg/d, Sigma-Aldrich Co., St. Louis, MO, U.S.A.) and a vehicle (saline; 1 ml/kg/d) were injected subcutaneously. Captopril (30 mg/kg/d, Nacalai Tesque Inc., Kyoto, Japan), telmisartan (3 mg/kg/d, LKT Laboratories Inc., St. Paul, MN, U.S.A.), and a vehicle (0.5% carboxymethyl cellulose; 1 ml/kg/d) were orally administered. All drugs were treated for 1 or 7 d.

Hemodynamic Analysis Hemodynamic analysis was performed at the day 2 under pentobarbital (50 mg/kg, intraperitoneally (i.p.)) anesthesia. The catheter filled with a heparin–saline solution was inserted into carotid artery with a small incision. Catheter was connected to MLT0670 disposable BP transducer (ADInstruments, Colorado Springs, CO, U.S.A.). Mean arterial pressure and heart rate were recorded with oral administration of captopril (30 mg/kg/d) or telmisartan (3 mg/kg/d).
measured using a ML117 BP Amp (ADInstruments), a ML825 PowerLab 2/25 (ADInstruments) system, and a Chart5 software (ADInstruments).

**Histological Analysis**  Hearts were excised and used for histological and biochemical examinations at the day 2 and day 8 under pentobarbital (50 mg/kg, i.p.) anesthesia. Isolated LV tissues were weighed. The LV tissues were fixed in 10% neutral buffered formalin for histological examination. Thin paraffin sections (4μm) were made and Azan staining was performed by a standard procedure. Fibrotic area was measured from 5 randomly selected area of LV sections at 40× magnification by using the Image J program (National Institutes of Health, Bethesda, MD, U.S.A.)\(^{13}\) Immunohistochemical staining for MMP-9 was performed by the peroxidase staining kit (LSAB2; Dako, Glostrup, Denmark) according to the instructions of the manufacturer’s. Mouse monoclonal antibody against MMP-9 (Daichi Fine Chemical Co., Ltd., Toyama, Japan) was used as the first antibody.

**Western Blotting**  Western blotting was performed as described previously.\(^{21}\) Briefly, LV tissues were homogenized in lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)-2Na, 1 mM ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na<sub>V</sub>O<sub>4</sub> 1 μg/ml leupeptin; Cell Signaling Technology Inc., Danvers, MA, U.S.A.) containing 1% protease inhibitor cocktail (Nacalai Tesque Inc.) and centrifuged. The supernatant was used for Western blotting. The proteins (60 μg) were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with 0.5% skim milk, the membranes were incubated with mouse monoclonal antibody against MMP-2 (Daichi Fine Chemical Co., Ltd.), rabbit polyclonal antibody against MMP-9 (Millipore Co., Billerica, MA, U.S.A.) and mouse monoclonal antibody against total actin (Sigma-Aldrich Co.). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG was used for the secondary antibody. Signal detection was achieved using the EZ-ECL western blotting detection reagents (Biological Industries Ltd., Kibbutz Beit Haemek, Israel) in the ATTO light capture system (AE-6972; ATTO Co., Tokyo).

**Statistical Analysis**  The results are presented as means±S.E.M. Statistical analyses were performed by one-way ANOVA followed by Bonferroni’s post-hoc test. A value of \(p<0.05\) was considered to be statistically significant.

**RESULTS**

**Effects of Captopril and Telmisartan on Isoprenaline-Induced LV Hypertrophy**  At the day 2, LV weight (LVW) in the ISO group did not change compared with the CONT group (data not shown). Table 1 shows the changes in body weight (BW), LVW, and LVW/BW ratio at the day 8. The BW in the ISO+CAP group significantly decreased compared with the CONT group (\(p<0.05\)). The LVW and LVW/BW in the ISO, ISO+CAP, and ISO+TEL groups significantly increased compared with the CONT group (LVW in ISO+CAP; \(p<0.05\), others; \(p<0.01\)). The BW, LVW and LVW/BW ratio in the ISO+CAP group significantly decreased compared with the ISO group (\(p<0.01\)). Telmisartan had no influence on the isoprenaline-induced biometrical changes. The LVW in the CAP group and the LVW and LVW/BW in the TEL group significantly decreased compared with the CONT group (\(p<0.05\)).

**Effects of Isoprenaline, Captopril, and Telmisartan on Hemodynamic Parameters**  The mean arterial pressure and the heart rate in the CONT, CAP, TEL, ISO, ISO+CAP, and ISO+TEL groups at the day 2 were measured by catheterization (Table 2). Isoprenaline, captopril and telmisartan decreased mean arterial pressure and increased heart rate compared with CONT, but the effects were not significant. The mean arterial pressure in ISO+CAP but not in ISO+TEL slightly decreased compared with ISO, and the heart rate in ISO+CAP increased compared with ISO, but these effects were not significant.

**Effects of Captopril and Telmisartan on Isoprenaline-Induced Myocardial Fibrosis in LV**  Myocardial fibrosis in LV at the day 8 was assessed by Azan staining (Fig. 1). No histological abnormality was observed in the LV sections from the CONT (A), CAP (B), and TEL (C). Myocardial fibrosis was observed in the sections from the ISO (D), ISO + CAP (E), and ISO + TEL (F). The percent of fibrotic area in the ISO+CAP significantly increased compared with the ISO (ISO+CAP; 25.0±1.3% vs. ISO; 14.1±2.7%, \(n=3\), \(p<0.01\), Fig. 1G). Telmisartan had no influence on the isoprenaline-induced fibrosis (16.0±1.9%, \(n=3\)).

**Effects of Captopril and Telmisartan on Isoprenaline-
Induced MMP-2 and MMP-9 Expressions in LV

Western blotting was used to investigate MMP-2 and MMP-9 expressions in LV. MMP-2 expression in the ISO at the day 2 did not change compared with the CONT (Figs. 2A, B). Isoproterenol significantly increased MMP-2 expressions compared with the CONT at the day 8 (ISO; 164 ± 11%, n = 6, p < 0.05, Figs. 2A, B). Captopril and telmisartan had no influence on the increased MMP-2 expression in the ISO (ISO/CAP; 199 ± 23%, ISO/TEL; 181 ± 20%, n = 6). On the other hand, MMP-9 expression in the ISO significantly increased compared with the CONT at the day 2 (ISO; 221 ± 24%, n = 6, p < 0.01, Figs. 3A, B). Captopril significantly enhanced the MMP-9 expression in the ISO (ISO/CAP; 645 ± 41%, n = 6, p < 0.01). Telmisartan had no influence on the isoproterenol-induced MMP-9 expression (208 ± 29%, n = 6) at the day 2. At the day 8, the MMP-9 expression in the ISO/CAP significantly increased compared with the CONT (255 ± 44%, n = 6, p < 0.01).

Immunohistochemical Localization of MMP-9 in LV

To clarify localization of MMP-9 expression, which significantly increased in the LV of the ISO/CAP at the day 2, we performed immunohistochemical staining (Fig. 4). MMP-9-positive interstitial cells were extensively observed in the LV section from the ISO/CAP (E). These MMP-9 positive-interstitial cells were rarely observed in the CONT (A), CAP (B), TEL (C), ISO (D) and ISO/TEL (F).

DISCUSSION

The present study demonstrated that both captopril and telmisartan do not suppress MMP-2 and MMP-9 expressions.
as well as LV fibrosis in isoprenaline-treated rats. It is also found that captopril rather promotes MMP-9 expression and myocardial fibrosis in isoprenaline-treated LV. To the best of our knowledge, this is the first report clarifying the effects of RAS blockade by captopril and telmisartan on MMP-2 and MMP-9 expressions during the development of isoprenaline-induced LV fibrosis.

In the present study, isoprenaline-treatment significantly increased the LVW and LVW/BW ratio. This feature is generally confirmative of the previous reports. The present study showed that telmisartan had no influence on the increased LVW/BW ratio by the isoprenaline-treatment. Leenen et al. reported that losartan, an AT1R antagonist, does not prevent the isoprenaline-induced cardiac hypertrophy in rats. Therefore, it is suggested that AT1R antagonists may not decrease the isoprenaline-induced cardiac hypertrophy on rats. In the present study, both captopril alone- and telmisartan alone-treatment decreased the LVW/BW ratio compared with the CONT. Omura et al. reported that delapril, an ACE inhibitor, and TCV-116, an AT1R antagonist, significantly decreased the LVW in normal rats. Therefore, angiotensin II might play a role in physiological cardiac growth in rats. It was reported that ACE inhibitors, such as trandolapril and ramipril, ameliorated the increased LVW/BW ratio by isoprenaline-treatment in rats. The present study showed that captopril significantly decreased the isoprenaline-induced LVW/BW ratio. However, the difference in the LVW/BW ratio between the ISO and ISO+CAP (0.24 mg/g) was almost same as the difference between the CONT and CAP (0.16 mg/g). Thus, it is suggested that captopril may decrease the normal cardiac development but have little influence on the isoprenaline-induced LV hypertrophy in rats. The previous report suggests that angiotensin II has a major function in maintaining isoprenaline-induced cardiac hypertrophy but not its induction.

Our data demonstrated that myocardial fibrosis is observed in the isoprenaline-treated LV at the day 8. This feature is consistent with the previous reports. In the present study, telmisartan had no influence on the isoprenaline-induced myocardial fibrosis. Leenen et al. reported that losartan increased the isoprenaline-induced LV fibrosis. On the other hand, Omura et al. reported that TCV-116 play a minor role in the isoprenaline-induced cardiac fibrosis. Therefore, it is suggested that AT1R antagonists may not suppress the isoprenaline-induced cardiac fibrosis in rats. In the present study, captopril rather promoted the isoprenaline-induced myocardial fibrosis. Ocaranza et al. reported that rats in the F2 generation with high plasma ACE activity developed more myocardial fibrosis by isoprenaline-treatment compared with rats with low plasma ACE activity. The authors also reported that collagen volumetric fraction and LV ACE activities were significantly correlated. On the other hand, Omura et al. reported that derapril has a minor role in the isoprenaline-induced myocardial fibrosis in rats. Furthermore, Grimm et al. demonstrated that ramipril had only little influence on the expression of ECM proteins in the isoprenaline-treated rats. Therefore, it is suggested that ACE inhibitors have only minimal or rather harmful effect on the isoprenaline-induced myocardial fibrosis in rats.

In the present study, the mean arterial pressure in ISO+CAP slightly decreased compared with ISO. In addition, the heart rate in ISO+CAP increased a little compared with ISO. However, these effects were not significant. Yeager and Iams reported that isoprenaline-induced myocardial damage is due to a relative myocardial hypoxia produced by arterial hypertension and myocardial hyperactivity. Therefore, decreasing arterial pressure and increasing heart rate induced by captopril might play a partial role in promoting isoprenaline-induced myocardial fibrosis.

MMP-2 and MMP-9, which belong to the gelatinase family, have been recognised to play a major role in the cardiac remodeling. It has been reported that the isoprenaline-treatment increased the MMP-2 and MMP-9 expressions in rat LV. In the present study, isoprenaline-treatment significantly increased the MMP-9 expressions at the day 2 and the MMP-2 expressions at day 8. It has been previously reported that the time course of the MMP-2 and MMP-9 induction is different. Tao et al. reported in mice that the MMP-9 activity increased 1 d after myocardial infarction, but the MMP-2 induction started within 4 d. We previously reported that isoprenaline-treatment for 4 d increased the MMP-2, but not the MMP-9 mRNA expression in rats. On the other hand, Ocaranza et al. demonstrated that the MMP-9 but not the MMP-2 activity showed a significant increase at 2 d after isoprenaline-treatment in rats. Thus, it is speculated that MMP-9 may act during the early phase and MMP-2 may act during the late phase in the development of cardiac remodeling.

We have provided evidence that captopril and telmisartan decreased myocardial fibrosis and the expressions of MMP-2 and MMP-9 in monocrotaline-treated rat RV. In the present study, captopril and telmisartan could not suppress isoprenaline-induced myocardial fibrosis and MMPs expressions. There are several reasons for this difference. First, monocrotaline-induced cardiac remodeling is caused by pressure overload. On the other hand, isoprenaline-induced cardiac remodeling is caused by β-adrenergic stimulation. Therefore, RAS may play a much more prominent role in pressure overload-induced cardiac remodeling than in β-adrenergic receptor stimulation-induced cardiac remodeling in rats. Second, monocrotaline-treatment induces RV remodeling, but we examined LV remodeling induced by isoprenaline in the present study. Leenen et al. reported that losartan inhibited RV fibrosis but not LV fibrosis in isoprenaline-treated rats. Therefore, the role of RAS in the development of RV and LV fibrosis may be different in rats. Third, Zhang et al. reported that olmesartan, an AT1R inhibitor, markedly suppressed isoprenaline-induced cardiac hypertrophy and collagen accumulation in mice. Thus, it is speculated that there is species-specificity in the effect of RAS on cardiac remodeling. These evidences suggest that RAS might have a minor role on the increase of MMPs expression and the enhancement of myocardial fibrosis in isoprenaline-induced cardiac remodeling in rat LV.

In the present study, captopril did not change the isoprenaline-induced MMP-2 expression at the day 8, but rather increased the isoprenaline-induced MMP-9 expressions at the day 2 and day 8. This study did not determine the mechanism by which captopril promoted the isoprenaline-induced MMP-9 expression in rat LV. Chronic treatment of isoprenaline induces β-adrenergic receptor desensitization. Yasunaga et al. suggested that the captopril-treatment increase...
β-adrenergic receptor in the isoprenaline-treated neonatal rat cardiomyocytes.\textsuperscript{21} On the other hand, Hsieh \textit{et al.} reported that bradykinin, which is degraded by ACE, induced the MMP-9 expression and cell migration in astrocytes.\textsuperscript{24} Thus, it is speculated that captopril may promote and prolong the isoprenaline-induced MMP-9 expressions through increasing cardiac β-adrenergic receptor or through inhibiting the degradation of bradykinin. Further study is needed to clarify the mechanism by which captopril promotes the isoprenaline-induced MMP-9 expression.

In the present study, immunohistochemical staining revealed that the extensive MMP-9-positive interstitial cells were observed in the LV section from the ISO+CAP at the day 2. Cardiac fibroblast is a major cell type in the myocardial interstitial cells and regulate cardiac remodeling, including myocardial fibrosis, through production of ECM and MMPs.\textsuperscript{25} Mukherjee \textit{et al.} reported that the MMP-9 promoter activation is colocalized in the myocardial macrophages, leukocytes, and fibroblasts after myocardial infarction of the MMP-9 reporter mice.\textsuperscript{26} In this study, captopril enhanced the isoprenaline-induced LV fibrosis. Thus, it is suggested that the enhancement of isoprenaline-induced MMP-9 expression by captopril in the interstitial cells, such as cardiac fibroblasts, promotes subsequent LV fibrosis in rats.

In conclusion, RAS blockade by captopril and telmisartan does not inhibit the isoprenaline-induced MMP-2 and MMP-9 expressions as well as myocardial fibrosis. Furthermore, captopril enhanced the isoprenaline-induced myocardial fibrosis as well as MMP-9 expression presumably in cardiac interstitial cells. These results suggest that RAS blockade is not always beneficial care to the cardiac remodeling.

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REFERENCES