Targeted Deletion of Matrix Metalloproteinase 2 Ameliorates Myocardial Remodeling in Mice With Chronic Pressure Overload

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Abstract—Matrix metalloproteinases (MMPs) play an important role in the extracellular matrix remodeling. Experimental and clinical studies have demonstrated that MMP 2 and 9 are upregulated in the dilated failing hearts and involved in the development and progression of myocardial remodeling. However, little is known about the role of MMPs in mediating adverse myocardial remodeling in response to chronic pressure overload (PO). We, thus, hypothesized that selective disruption of the MMP 2 gene could ameliorate PO-induced cardiac hypertrophy and dysfunction in mice. PO hypertrophy was induced by transverse aortic constriction (TAC) in male MMP 2 knockout (KO) mice (n=10) and sibling wild-type (WT) mice (n=9). At 6 weeks, myocardial MMP 2 zymographic activity was 2.4-fold increased in WT+TAC, and this increase was not observed in KO+TAC, with no significant alterations in other MMPs (MMP 1, 3, 8, and 9) or tissue inhibitors of MMPs (1, 2, 3, and 4). TAC resulted in a significant increase in left ventricular (LV) weight and LV end-diastolic pressure (EDP) with preserved systolic function. KO+TAC mice exerted significantly lower LV weight/body weight (4.2±0.2 versus 5.0±0.2 mg/g; P<0.01), lung weight/body weight (4.9±0.2 versus 6.2±0.4 mg/g; P<0.01), and LV end-diastolic pressure (4±1 versus 10±2 mm Hg; P<0.05) than WT+TAC mice despite comparable aortic pressure. KO+TAC mice had less myocyte hypertrophy (cross-sectional area; 322±14 versus 392±14 μm²; P<0.01) and interstitial fibrosis (collagen volume fraction; 3.3±0.5 versus 8.2±1.0%; P<0.01) than WT+TAC mice. MMP 2 plays an important role in PO-induced LV hypertrophy and dysfunction. The inhibition of MMP 2 activation may, therefore, be a useful therapeutic strategy to manage hypertensive heart disease. (Hypertension. 2006;47:711-717.)

Key Words: hypertrophy ■ heart failure ■ fibrosis ■ extracellular matrix ■ hypertension, experimental ■ myocardium

Left ventricular (LV) hypertrophy is an adaptive process that compensates for pressure overload (PO) caused by hypertension or valvular heart disease, such as aortic stenosis. This remodeling process consists of hypertrophic changes of cardiac myocytes and abnormalities of the extracellular matrix (ECM) network, which are both responsible for changes in systolic and diastolic function.¹

The dynamic synthesis and breakdown of ECM proteins play an important role in adverse myocardial remodeling. In particular, the increased expression and activation of the matrix metalloproteinases (MMPs) have been shown in various forms of heart failure and implicated in the process of myocardial remodeling that is characteristic of developing heart failure.² Despite a number of studies implicating MMPs in cardiac pathophysiology, little is known about the role of MMP in the development of myocardial remodeling in response to chronic PO. MMP 2 and 9 expression has been shown to be enhanced in pressure-overloaded cardiac hypertrophy in spontaneously hypertensive rats³ and in Dahl salt-sensitive hypertensive rats.⁴ Similar upregulation of MMPs has been also observed in human pressure-overloaded hearts because of aortic stenosis.⁵ Recently, Heymans et al⁶ have demonstrated that MMP 9 is involved in cardiac remodeling associated with hypertension. However, MMP-9 is mainly expressed in such infiltrating inflammatory cells as neutrophils and macrophages.⁷ Conversely, MMP 2 is ubiquitously distributed in cardiac myocytes and fibroblasts.⁸ Therefore, MMP 2 may also play an important role in the development and progression of myocardial remodeling in response to PO. However, no previous studies have yet determined the pathophysiological significance of MMP 2 in this disease state.

In the present study, we evaluated the effects of a targeted deletion of the MMP 2 gene on both LV structural and...
functional alterations during pressure-overloaded cardiac hypertrophy. To ensure selective and long-term complete inhibition of MMP 2, we used MMP 2 knockout (KO) mice.9–11 The most effective way to evaluate the contribution of the specific MMP and obtain the direct evidence for a role of MMP is through gene manipulation instead of MMP inhibitor.

Methods

Experimental Animals

The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. We used the progeny of homozygous breeding pairs of C57BL/6J mice with targeted disruption of MMP 2 ranging in age from 11 to 14 weeks old.10 The mutation heterozygous mice were obtained by crossing the chimeras to C57BL/6J mice. Heterozygotes were backcrossed to C57BL/6J 1 to 5 times and then crossed to obtain the mutation homozygous mice. The original breeding pairs used to develop the mice for this study were obtained from Dr Shigeyoshi Itohara (Laboratory for Behavioral Genetics, RIKEN, Tsukuba, Japan).

Transverse Aortic Constriction

Transverse aortic constriction (TAC) was performed in male MMP 2 KO and sibling wild-type (WT) mice as described previously.12 Briefly, after anesthetizing by tribromoethanol/amylene hydrate (Avertin; 2.5% weight/volume, 8 µL/g IP), mice were intubated and ventilated, and a thoracotomy was performed via the second intercostal space at the left upper sternal border. The transverse aortic arch was ligated between the innominate and left common carotid arteries with an overlying 28-gauge needle, which, after removal of the needle, left a reproducible discrete region of stenosis. Sham-operated mice underwent a similar procedure without ligation of the aorta.

Tail clips and a PCR protocol to confirm the genotype were performed by a group of investigators. Next, TAC was induced in these mice by another subset of investigators, who were not informed of the genotyping results. This assignment procedure was performed using numeric codes to identify the animals.

MMPs and Tissue Inhibitors of MMPs

First, the myocardial MMP levels, including MMP 2 and MMP 9, were determined in the left ventricle (LV) using gelatin zymography as described previously.13 The LV myocardial samples were homogenized (~50–s bursts) in 1 mL of an ice-cold extraction buffer containing cacodylic acid (10 mMol/L), NaCl (0.15 mol/L), ZnCl₂ (20 mMol/L), Na₂HPO₄ (1.5 mMol/L), and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4°C, 10 minutes, 10 000 g) and the supernatant decanted and saved on ice. The pH levels of the samples were adjusted to 7.5 using Tris (1 mol/L). The final protein concentration of the myocardial extracts was determined using a standardized colorimetric assay. The extracted samples were then aliquoted and stored at ~80°C until the time of assay. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin under nonreducing conditions. The myocardial extracts at a final protein content of 5 µg were loaded onto the gels using a 3:1 sample buffer (10% SDS, 4% sucrose, 0.25 mol/L Triton-Cl, and 0.1% bromophenol blue <pH 6.8>). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), whereas the running buffer temperature was maintained at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 minutes each, rinsed in water, and incubated for 24 hours in a substrate buffer at 37°C (50 mMol/L Tris-HCl, 5 mMol/L, CaCl₂, and 0.02% Na₃ [pH 7.5]). After incubation, the gels were stained with Coomassie brilliant blue R-250. The zymograms were digitized, and the size-fractionated bands, which indicated the MMP proteolytic levels, were measured by the integrated optical density in a rectangular region of interest.

Next, the mRNA levels of myocardial MMPs including MMP 1, 2, 3, 8, and 9 as well as tissue inhibitors of MMPs (TIMPs) including TIMP 1, 2, 3, and 4 were determined by multiprobe ribonuclease protection assay (RiboQuant, PharMingen). Each value was normalized to that of glyceraldehydes-3-phosphate-dehydrogenase in each template set as an internal control, followed by calculation as a ratio to WT + Sham. The amount of tissue was limited in mice and, thus, tissue needed to be divided so that all of the biochemical analyses could be performed.

Echocardiographic and Hemodynamic Measurements

Echocardiographic studies were performed under light anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% weight/volume, 8 µL/g IP) and spontaneous respiration as described previously.14 A 2D parasternal short-axis view of the LV was obtained at the level of the papillary muscles. In general, the best views were obtained with the transducer lightly applied to the mid-upper left anterior chest wall. The transducer was then gently moved cephalad or caudal and angulated until desirable images were obtained. After confirming that the imaging was on axis (based on roundness of the LV cavity), 2D targeted M-mode tracings were recorded at a paper speed of 50 mm/s. Two consecutive heartbeats of each frame were analyzed to measure the wall thickness and end-diastolic (EDD) and end-systolic (ESD) internal dimensions of the LV. LV fractional shortening (FS) was calculated as LV EDD minus LV ESD normalized for LV EDD and was taken as an index of LV systolic performance. Echocardiographic LV mass was calculated according to the standard cube formula as described previously.15 Our previous validation study has shown that the intraobserver and interobserver variabilities of our echocardiographic measurements for LV cavity dimensions and FS were small, and measurements made in the same animals on separate days were highly reproducible.14 Next, a 1.4-Fr micromanometer-tipped catheter (Millar) was inserted into the right carotid artery and then was advanced into the LV to measure the LV pressures. Arterial blood pressure and heart rate were also measured with the use of a noninvasive tail-cuff system (BP-98A, Softron).16

Organ Weight and Histopathology

After in vivo echocardiographic and hemodynamic studies, the heart and lung were excised, and their weights were determined. The heart was dissected into the right and left ventricles, including the septum. From the mid-LV transverse sections, 5-µm sections were cut and stained with hematoxylin/eosin and Masson’s trichrome to determine the myocyte cross-sectional area and collagen volume fraction. To measure the myocyte cross-sectional area, each section was photographed using a microscope and magnified (final magnification: ×750). Connective tissue and muscle areas were identified, and the profile margin of 30 to 40 myocytes cut into cross-sections was manually traced and digitized. The digitized profiles were transferred to a personal computer that calculated the area. Three to 4 fields were randomly selected from 2 to 3 coronal sections of each heart. Thus, ~100 to 200 myocytes were measured for each animal, and the mean myocyte cross-sectional area was calculated.17 Collagen volume fraction was measured at ~5 to 7 fields for each heart.18 Within each field, segments representing connective tissue and myocyte were identified and manually traced by using a digitizing pad with a computer to calculate the traced area. Collagen volume fraction was then calculated for the heart as the sum of all of the connective tissue areas divided by the sum of all of the connective tissue and muscle areas in all fields. Collagen surrounding intramyocardial coronary arteries was excluded from the analysis.

Statistical Analysis

All of the data are expressed as the mean±SEM. Between-group comparisons of the means were performed by 1-way ANOVA, followed by t tests. The Bonferroni’s correction was done for multiple comparisons of the means.
Results

MMPs and TIMPs

At 6 weeks of TAC, the zymographic MMP 2 levels increased by 2.4-fold compared with WT/Sham mice (Figure 1). As expected, MMP 2 activity was not detected in KO/Sham and KO/TAC mice. Importantly, the MMP 9 zymographic levels did not differ between WT/TAC and KO/TAC.

Again, the MMP 2 mRNA levels significantly increased in the WT/TAC compared with WT/Sham (Figure 2). This increase was not observed in KO/TAC. These results were consistent with those observed in gelatin zymography (Figure 1). Other MMPs, including MMP 1, 3, 8, and 9, were not altered in these mice (Figure 2). The changes of TIMPs (TIMP 1, 2, 3, and 4) were also comparable between WT/TAC and KO/TAC.

Echocardiography

The presence or absence of MMP 2 gene did not affect baseline heart rate or echocardiographic parameters in Sham mice (Table 1). TAC significantly increased LV wall thickness (Table 1) and echocardiographic LV mass (Figure 3) without affecting LV diameters or FS in WT/TAC. These LV hypertrophic changes in WT/TAC were significantly ameliorated in KO/TAC.

Hemodynamics

LV systolic pressure was markedly elevated by TAC (Table 2). However, there was no significant difference in LV systolic pressure between WT/TAC and KO/TAC mice. LV EDP increased significantly in WT/TAC mice, and this increase was significantly attenuated in KO/TAC mice.
Organ Weights

In agreement with echocardiographic LV mass (Figure 3), TAC increased LV weight in WT mice (Figure 4A). Furthermore, in accordance with LV EDP, lung weight/body weight, indicative of pulmonary congestion, increased significantly in WT+TAC (Figure 4B). In KO+TAC, both increased LV and lung weights were significantly reduced.

Histopathology

Masson’s trichrome staining showed the increase in myocyte size and interstitial collagen volume fraction in WT+TAC mice compared with WT+Sham mice (Figure 5). The selective disruption of the MMP 2 gene significantly ameliorated myocyte hypertrophy and interstitial fibrosis by TAC. Similarly, picrosirius staining also demonstrated collagen deposition in the interstitial areas in LV sections from WT+TAC mice and the amelioration of interstitial fibrosis in KO+TAC mice (data not shown).

Discussion

In the present study, we demonstrated that the selective disruption of the MMP 2 gene ameliorated LV remodeling, such as myocyte hypertrophy and interstitial fibrosis in TAC mice, thus providing direct evidence that MMP 2 is involved in mediating PO-induced cardiac hypertrophy. These beneficial effects of MMP 2 inhibition occurred without affecting hemodynamics.

Upregulation of MMP 2 in PO Hypertrophy

The present study demonstrated that the MMP 2 gene expression and gelatinolytic activities were upregulated in the myocardium during PO (Figures 1 and 2). These findings, coupled with past reports, suggest that an increase in myocardial MMP levels is a fairly uniform event in myocardial remodeling. Although the mechanisms responsible for this activation remain to be determined, cellular constituents of cardiac muscle, including fibroblasts, inflammatory cells, and myocytes, are known to be capable of expressing MMP 2 in response to specific stimuli, including mechanical stress.

Role of MMP 2 in PO Hypertrophy

Alterations in the expression and activity of MMP 2 have been demonstrated in a number of pathophysiological conditions, such as myocardial infarction (MI) and heart failure. Our previous study and a recent study by Matsumura et al have demonstrated that the inhibition of MMP 2 activity improves the survival rate after acute MI by preventing cardiac rupture and delays after MI remodeling. A recent study by Wang et al has shown that cardiac-specific, constitutively active MMP 2 expression leads to impaired contraction and diminished responses to inotropic stimulation, indicating that MMP 2 can directly impair cardiac function in the absence of superimposed injury.

Broad-spectrum pharmacological inhibition of MMPs significantly attenuated myocardial remodeling associated with chronic volume overload or hypertension. However, the most effective way to evaluate the contribution of the specific MMP and to obtain the direct evidence for a role of MMP in myocardial remodeling and failure is through such gene manipulation as that used in the present study. As expected, no MMP 2 expression was observed in the myocardium from KO mice in this study (Figures 1 and 2). Thus, the present study could investigate the effects of selective disruption of the MMP 2 gene on the development of myocardial hypertrophy induced by PO.

The most striking finding of the present study was the inhibition of myocardial hypertrophy in MMP 2 KO mice.
under PO (Figures 3 through 5). Our previous studies demonstrated the beneficial effects of MMP 2 deletion also on postinfarct LV remodeling and failure. Similar to the present study, both myocyte hypertrophy and interstitial collagen accumulation were ameliorated in the MMP 2–deficient mice after MI. Moreover, the present study is consistent with previous studies from other laboratories, which demonstrated the involvement of myocardial MMP in heart failure. Therefore, the present study builds on these past reports by demonstrating that MMP inhibition attenuated myocardial remodeling that occurred also under PO.

The present study demonstrated that increased MMP 2 activity was associated with the interstitial fibrosis in pressure-overloaded LV and the selective inhibition of MMP 2, indeed, ameliorated these changes (Figure 5). Theoretically, an increase in MMP activity would result in a decrease in the MMP substrate, collagens, whereas an inhibition of MMP would result in an increase in collagens. However, in agreement with previous studies, an increase in MMP 2 activity was accompanied by increased fibrosis in our model of LV hypertrophy, which is probably because of the direct proteolysis of myocardial matrix components, as well as by facilitating a profibrotic response. In fact, the selective disruption of the MMP 2 gene did attenuate interstitial fibrosis. Although the present study could not provide the definite explanation for these paradoxical findings, this might be because of the fact that the total ECM collagen content is a complex function of both synthesis and degradation.

In addition to interstitial fibrosis, myocyte cellular hypertrophy was also ameliorated by the selective blockade of the MMP 2 gene (Figure 5), suggesting that myocardial induction of MMP 2 is involved in the development of myocyte hypertrophy during PO. Recently, Heymans et al demonstrated that MMP 9 gene inactivation reduced hypertrophic changes in cardiac myocytes during acute PO. These results suggest that there is an intimate link between MMPs and the myocyte hypertrophic process, which might be mediated, at least in part, by the tissue infiltration of inflammatory cells. However, the precise role of MMPs in the development of myocyte hypertrophy has not been fully explored. MMPs may be involved in a complex myocyte–matrix interaction, because the basement membrane components, collagen IV and laminin, are the substrates for MMP 2 and MMP 9. Thus, increased MMP 2 activity within the myocardium can contribute to the discontinuity of the basement membrane, thereby disrupting the normal myocyte-matrix interface. The findings that MMP inhibition limited the degree of myocyte hypertrophy raise an issue requiring additional studies.

**Limitations**

There are several issues to be acknowledged as a limitation in this study. First, although in vivo assessment of LV function with echocardiography is feasible and reproducible in the mouse, it might still be difficult to interpret the indices of LV function. However, our validation study has shown that the intraobserver and interobserver variabilities of our echocardio-
Chronic PO results in an initial cardiac hypertrophic response followed by progressive failure. Our findings provide the first evidence that MMP 2 is involved in mediating the attenuation of LV hypertrophy and the resultant effects of MMP 2 inhibition occurred in the absence of a decrease in systemic blood pressure. These findings suggest that the early institution of MMP 2 inhibition may have use in preventing the development of maladaptive myocardial remodeling in response to hypertension.

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