Activation of Matrix Metalloproteinases Precedes Left Ventricular Remodeling in Hypertensive Heart Failure Rats

Its Inhibition as a Primary Effect of Angiotensin-Converting Enzyme Inhibitor

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Background — Matrix metalloproteinases (MMPs) are activated in dilated failing hearts, and angiotensin-converting enzyme (ACE) inhibition prevents left ventricular (LV) dilatation. However, it remains unclear whether activation of MMPs precedes or is secondary to LV remodeling, and an effect of ACE inhibition on MMPs is unknown.

Methods and Results — Dahl salt-sensitive rats fed a high-salt diet from 8 weeks served as the hypertensive heart failure (HF) model. LV echo, histological study, measurement of mRNA levels, and gelatin zymography were performed before (at 23 weeks) and after (at 26 weeks) the development of LV dilatation and pulmonary edema. The same procedures were conducted in the HF model rats treated with a subdepressor dose of ACE inhibitor (enalapril 5 mg · kg\(^{-1}\) · d\(^{-1}\)) from 9 weeks. Rats fed on normal chow served as age-matched controls. In the untreated HF model rats, gene expression of MMP-2 and MMP-9 and tissue gelatinase activity were elevated at 23 weeks without LV dilatation. LV dilatation, LV systolic dysfunction, and pulmonary edema occurred at 26 weeks, with further enhancement of the expression and activity of MMPs. ACE inhibition prevented such geometrical and functional deterioration. The gene expression and activity of MMPs were suppressed by ACE inhibition at 23 weeks without a decrease in blood pressure, and the suppressive effects continued at 26 weeks.

Conclusions — MMPs are likely to trigger and promote LV remodeling, and ACE inhibition directly exerts inhibitory effect on MMPs, leading to the prevention of LV remodeling and dysfunction. (Circulation. 2004;109:2143-2149.)

Key Words: remodeling ■ heart failure ■ hypertension

Left ventricular (LV) remodeling is associated with poor prognosis in patients with heart failure (HF), and its attenuation is considered a principal benefit of ACE inhibition. However, the mechanisms of LV remodeling or of the benefit of ACE inhibition remain to be elucidated.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes and play an important role in degradation of extracellular matrix. Gene expression and gelatinolytic activity of MMPs in the left ventricle were enhanced in animal and human failing hearts. The previous studies assessed the expression and activity of MMPs after LV dilatation occurred, and preventive pharmacological inhibition of MMPs resulted in incomplete suppression of LV remodeling in animal HF models. There is no doubt about the crucial roles of MMPs in promoting LV remodeling; however, it remains unclear whether activation of MMPs precedes or follows LV dilatation. Previous in vitro studies showed that ACE inhibition attenuated MMP activity and long-term ACE inhibition decreased MMP activity with attenuation of LV dilatation in animal studies. However, it is unclear whether the suppression of MMP activity by ACE inhibition is causative of or secondary to prevention of LV dilatation in vivo. We aimed to investigate gene expression and gelatinolytic activity of MMPs just before and after the development of LV remodeling in a hypertensive HF model and to elucidate the effects of ACE inhibition in vivo. To exclude depressor effects on MMP gene expression and activity, a subdepressor dose of ACE inhibitor was administered.

Methods

This study conforms to the guiding principles of Osaka University Graduate School of Medicine with regard to animal care and to the Position of the American Heart Association on Research Animal Use.

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Study Subjects
Male Dahl salt-sensitive rats fed 8% NaCl from 8 weeks were used as a hypertensive HF model (n=24). We randomly selected 12 of the 24 rats to give the ACE inhibitor enalapril from age 9 weeks (5 mg kg\(^{-1}\) d\(^{-1}\)). The others were given placebo gavages [ACEI(−) group]. The dose of enalapril was determined according to our previous study.\(^{16}\) The others were given placebo [ACEI(−) group]. The rats fed 0.3% NaCl were normotensive and served as age-matched control (N group; n=12). We randomly selected 6 rats from the N group, 4 rats from the ACE(−) group, and 4 rats from the ACE(+)+ group to study their characteristics at 23 weeks. The other rats in the 3 groups were studied at 26 weeks. This protocol was decided after our preliminary study, which demonstrated that LV dilatation and systolic dysfunction were absent at 23 weeks and present at 26 weeks in this model. Systolic blood pressure was measured with a tail-cuff system (BP-98A, Softron).

Doppler Echo Study
The rats were anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and transthoracic echocardiography was conducted before the harvest of the heart at 23 or 26 weeks.\(^{16}\)

Tissue Sampling and Pathophysiological Studies
After the echo study and adequate anesthesia, blood was sampled from the right carotid artery for measurement of plasma angiotensin II,\(^{17}\) the lung and the heart were harvested, and the lung and the LV were weighed. The LV weight corrected for body weight was determined as LV mass index.\(^{15}\) The LV myocardium was immediately placed in liquid nitrogen and stored at –80°C for the measurement of mRNA levels and in vitro zymography. Samples for in situ zymography and immunohistochemistry were embedded in Tissue-Tek OCT compound (Sakura Finetechical Co) and frozen on dry ice. The rest of the left ventricle was fixed with a phosphate-buffered 10% formalin solution for 48 hours. The specimens were embedded in paraffin, and transverse sections 2 μm thick were stained with azan Mallory stain to evaluate interstitial fibrosis using the percent area of fibrosis.\(^{15}\)

Quantification of Gene Expression
The mRNA level in the left ventricle was quantified by real-time quantitative polymerase chain reaction with Prism 7700 Sequence Detector (Perkin-Elmer Corp.).\(^{17}\) Sequences of all oligonucleotides used as forward primers, reverse primers, and detection probes are summarized in Table 1. The amounts of each measured mRNA were corrected for those of GAPDH mRNA.

Immunohistochemistry
Serial cryostat transverse sections 4 μm thick were fixed in acetone for 10 minutes, air-dried, and stained by the indirect immunohistochemical method using rabbit anti-rat MMP-9 polyclonal antibody (1:500 dilution, TP-221P Torrey Pines Biolabs), rabbit anti-mouse MMP-2 monoclonal antibody (1:40 dilution, F-73 Fuji Chemical Co), or mouse anti-rat macrophage monoclonal antibody (1:50 dilution, clone Ki-M2R, BMA Biomedicals AG) as previously described.\(^{18}\)

Zymography
The in vitro gelatin zymography was conducted as previously described.\(^{18}\) The in vitro zymography may overestimate net functional activity of MMPs because MMP–tissue inhibitor of matrix metalloproteinase (TIMP) complexes are dissociated during separation in SDS-PAGE.\(^{19}\) Therefore, the film in situ gelatin zymography that allows identification of net functional gelatinolytic activity in tissues\(^{19}\) was conducted with a commercially available kit (MMP in situ Zymo-Film, Wako Pure Chemical Industries, Ltd.).\(^{18,19}\) The in situ zymography was also conducted in the presence of TIMP-2, EDTA, PMSF, and E-64 (Figure 1). The gelatinolytic activity was inhibited by TIMP-2 and EDTA dose-dependently but not by PMSF. Thus, the observed gelatinolytic activity was not attributed to serine proteases such as plasmin or plasminogen activator. Because E-64 slightly inhibited the activity, cysteine proteases may contribute in part to the gelatinolysis. However, the gelatinolysis was completely inhibited by TIMP-2 and EDTA and may be attributed primarily to MMP activity.

Statistical Analysis
Results are expressed as mean±SEM. The serial data were analyzed by 2-way ANOVA. Differences at specific stages among groups were assessed using 1-factor ANOVA and Bonferroni/Dunn’s test. A probability value of P<0.05 was considered statistically significant.

Results

**Hemodynamics**
Systolic blood pressure was higher in the ACEI(−) group than in the N group at 23 and 26 weeks, and ACE inhibition did not significantly reduce blood pressure (Table 2). The E/A ratio of the transmitral flow velocity curves was lower at 23 weeks in the ACE(−) and ACE(+) groups than in the N group, and there was no difference in the ratio of lung weight to body weight among the 3 groups at this stage (Figure 2 and Table 2). E/A ratio and ratio of lung weight to body weight increased from 23 to 26 weeks in the ACEI(−) group, indicating increased LV end-diastolic pressure and pulmonary edema.\(^{15,16}\) The increases were prevented by ACE inhibition, indicating that long-term ACE inhibition prevented the transition to congestive HF. Plasma angiotensin II level was not different at 26 weeks among the 3 groups (N, 15±2; ACEI(−), 19±10; ACEI(+), 27±7 pg/mL).

**LV Geometry, Structure, and Function**
LV end-diastolic diameter, fractional shortening, and midwall fractional shortening were not different at 23 weeks among the 3 groups. At 26 weeks, LV end-diastolic diameter was larger and fractional shortening and midwall fractional shortening were lower in the ACE(−) group than in the N group, and ACE inhibition prevented LV dilatation and systolic dysfunction (Figure 2 and Table 2).

**LV mass, mass index, and area of fibrosis were greater in the ACE(−) group than in the N group at 23 and 26 weeks. ACE inhibition lessened their increases at both stages (Table 2).**

**Gene Expressions of MMPs, TIMPs, and Collagens**
The mRNA levels of MMP-2 and MMP-9 were higher at 23 weeks in the ACE(−) group than in the N group, whereas there was no difference in MMP-13 (rat collagenase) and MMP-14 mRNA levels (Figure 3). Gene expression of MMP-2, MMP-9, MMP-13, and MMP-14 was significantly enhanced from 23 to 26 weeks in the ACE(−) group, and their mRNA levels at 26 weeks were higher than in the N group. ACE inhibition normalized their gene expression at both 23 and 26 weeks.

The mRNA levels of TIMP-1, TIMP-2, collagen type I, and collagen type III increased significantly in the ACE(−) group at 23 and 26 weeks compared with the N group (TIMP-1, 4.4-fold at 23 weeks, 7.6-fold at 26 weeks; TIMP-2, 2.0-fold at 23 weeks, 2.7-fold at 26 weeks; collagen type I, 2.9-fold at 23 weeks, 3.6-fold at 26 weeks; collagen type III, 2.7-fold at 23 weeks, 2.7-fold at 26 weeks). These increases were prevented by ACE inhibition.
Gelatinolytic Activity by In Vitro and In Situ Zymography

Activity of 72-kDa gelatinase (MMP-2) increased at 23 weeks in the ACE(−) group and was further promoted at 26 weeks (Figure 4). Gelatin lysis in the region of 92-kDa gelatinase (MMP-9) was enhanced only at 26 weeks in the ACE(−) group. In the ACE(+) group, activity of 72-kDa gelatinase decreased at 23 and 26 weeks compared with the ACE(−) group. ACE inhibition also suppressed gelatinolytic activity at the 92-kDa region at 26 weeks. As shown in Figure 5, net functional gelatinolytic activity as assessed by the in situ zymography increased in the ACE(−) group at 23 weeks and was further enhanced at 26 weeks. The gelatinolytic activity was attenuated by ACE inhibition at 23 and 26 weeks.

Immunohistochemistry

Macrophage infiltration was prominent in the ACE(−) group compared with the N group at 23 and 26 weeks (Figure 6A) and was suppressed by long-term ACE inhibition. At 26 weeks, staining for macrophages colocalized with that for MMP-2 and MMP-9 in the ACE(−) rats (Figure 6B); however, the areas positive for MMP-2 and MMP-9 included those without staining for macrophages.

### TABLE 1. Sequences of All Oligonucleotides Used as Forward Primers, Reverse Primers, and Detection Probes

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<th>mRNA</th>
<th>Oligonucleotide Sequence</th>
<th>GenBank Locus</th>
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<td>GAPDH</td>
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<td>Probe</td>
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<td>Probe</td>
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The numbers in parentheses indicate the position in the reported sequences.
Discussion

Increases in mRNA levels of MMP-2 and MMP-9 and enhancement of gelatinase activity preceded LV dilatation in the hypertensive HF model. Long-term administration of ACE inhibitor suppressed the gene expression and activity of MMPs at the predilatation stage independently of its depressor effect and prevented LV remodeling, systolic dysfunction, and the transition to congestive HF.

Role of MMPs in LV Remodeling

Changes in expression and activity of several MMPs have been identified in human failing myocardium. However, there is limited information about temporal changes in their expression and activity during the progression of LV remodeling. Previous studies demonstrated that expression of MMPs was enhanced during the progression of LV dilatation in animal HF models, which is compatible with our results. However, the previous studies observed changes in the expression only after the development of LV dilatation. The present study expanded the previous studies by demonstrating that enhancement of gene expression of MMP-2 and MMP-9, activation of 72-kDa gelatinase and promotion of gelatinolytic activity preceded LV dilatation. This result suggests that enhancement of gelatinase activity plays an important role as a trigger of LV remodeling and an accelerator. Iwanaga et al recently demonstrated that total activity of MMP-2 remained unchanged before LV dilatation. However, they did not measure the activity at a later stage just before the initiation of LV dilatation, and this may be responsible for their conclusion being different from ours.

The present study showed that enhancement of MMP-2 activity without activation of MMP-9 at 23 weeks was not associated with LV dilatation and that LV dilatation at 26 weeks was accompanied with concomitant activation of MMP-2 and MMP-9. Ducharme et al showed that the

| TABLE 2. Hemodynamic and Pathophysiological Results |
|---------------------------------|-----------------|-----------------|-------------------|-----------------|-------------------|
|                                | 23 Weeks        | 26 Weeks        |
|                                | N Group         | ACE(−)          | ACE(+)           | N Group         | ACE(−)           | ACE(+)           |
|                                | (n=6)           | (n=4)           | (n=4)            | (n=6)           | (n=8)            | (n=8)            |
| Systolic blood pressure, mm Hg | 128±3           | 212±7*          | 198±16*          | 128±3           | 221±6†           | 209±4†           |
| Body weight, g                | 415±8           | 407±7           | 428±18           | 422±8           | 371±11†          | 439±10†‡         |
| E/A                            | 1.9±0.2         | 1.2±0.1*        | 1.1±0.1*         | 2.1±0.3         | 4.4±1.1†         | 1.2±0.1†         |
| Lung wt/body wt, mg/g          | 3.3±0.1         | 3.9±0.1         | 3.9±0.1          | 3.3±0.1         | 11.3±2.1†        | 3.7±0.1‡         |
| LV end-diastolic diameter, mm  | 8.9±0.1         | 9.0±0.3         | 9.3±0.2          | 8.9±0.2         | 10.5±0.3†        | 6.5±0.2‡         |
| Fractional shortening, %       | 33±2            | 33±4            | 37±1             | 32±2            | 25±1†            | 34±2‡            |
| Midwall fractional shortening, % | 18±1            | 17±1            | 18±1             | 19±1            | 13±1†            | 16±1†            |
| LV mass, g                    | 0.84±0.01       | 1.26±0.05*      | 1.15±0.03§       | 0.82±0.01       | 1.49±0.04†       | 1.24±0.03†‡       |
| LV mass index, mg/g            | 2.02±0.04       | 3.10±0.08*      | 2.68±0.05§       | 1.95±0.02       | 4.03±0.14†       | 2.85±0.08†‡       |
| Area of fibrosis, %            | 2.5±0.1         | 7.0±1.6*        | 3.1±0.3         | 4.5±0.3         | 9.2±2.0†         | 3.2±0.2‡         |

Values are expressed as mean±SEM.

*P<0.05 vs N group at 23 weeks.
†P<0.05 vs N group at 26 weeks.
‡P<0.05 vs ACE(−) group at 26 weeks.
§P<0.05 vs ACE(−) group at 23 weeks.
genetic deletion of MMP-9 attenuated LV dilatation after myocardial infarction despite the increased expression of MMP-2. Longo et al.\textsuperscript{23} recently showed that MMP-2 and MMP-9 work in concert to induce aortic aneurysm and that a lack of either MMP prevented it. Thus, the present study and others suggest that a concomitant activation of several MMPs, including MMP-2 and MMP-9, rather than an activation of a specific MMP promotes ventricular remodeling as well as vascular remodeling. At 23 weeks, an increase in mRNA level of MMP-9 was not associated with activation of MMP-9 as assessed by the in vitro zymography. Thus, posttranscriptional or posttranslational modulation of MMP-9 expression may be present at 23 weeks.

Macrophages significantly infiltrated into the myocardium in the HF rats, which is compatible with previous studies.\textsuperscript{24,25} Macrophages play a crucial role in the production and secretion of MMPs in atherosclerotic regions.\textsuperscript{26,27} Immunohistochemical staining for macrophages colocalized with that for MMP-2 and MMP-9, but staining for MMP-2 and MMP-9 was not necessarily adjacent to that for macrophages in this study. Thus, the present result suggests that macrophages contribute to production and secretion of MMP-2 and MMP-9 but are not the sole cell type to express MMPs.

Gene expression of TIMPs was enhanced with that of MMPs, but the film in situ zymography demonstrated an increase in gelatinolytic activity. Thus, expression of MMPs was likely to surpass that of TIMPs. However, in addition to inhibitory effects of TIMPs on MMPs, TIMPs may actually stabilize, localize, and even activate MMPs under certain conditions.\textsuperscript{28} Future studies focusing on the role of TIMPs in LV remodeling are required.

**Effect of ACE Inhibition on MMP Activity**

The present study demonstrated that long-term ACE inhibition attenuated LV remodeling and prevented the transition to congestive HF in the hypertensive HF model without changes in plasma angiotensin II level. Thus, the effects of ACE inhibition were most likely attributable to suppression of the cardiac renin-angiotensin system.

Previous studies showed that ACE inhibition attenuated MMP activity in vitro,\textsuperscript{7,11} and decreased gene expression and gelatinolytic activity of MMPs in association with the prevention of LV remodeling in animal studies.\textsuperscript{12–14} However, none of in vivo results fully showed whether the effect of ACE inhibition on MMPs was a cause for or a result of the prevention of the LV geometrical and hemodynamic deteri-
oration. In this study, the administration of ACE inhibitor at a subdepressor dose suppressed gene expression and gelatinolytic activity of MMPs even at the predilatation stage and prevented LV dilatation in the hypertensive HF model. Thus, this study expanded the previous studies by demonstrating that the suppressive effect of ACE inhibition on gelatinase activity is its primary action in vivo. However, a question may be still raised as to whether LV dilatation was prevented or only delayed. We conducted an additional experiment to reveal that LV end-diastolic diameter, fractional shortening, or LV mass index calculated by echocardiography as previously described did not change at least until 31 weeks (LV end-diastolic diameter, 9.0±0.2 mm at 23 weeks, 8.9±0.1 mm at 26 weeks, 9.2±0.2 mm at 31 weeks; fractional shortening, 36±2% at 23 weeks, 36±1% at 26 weeks, 35±1% at 31 weeks; LV mass index, 2.82±0.16 mg/g at 23 weeks, 2.71±0.14 mg/g at 26 weeks, 2.76±0.20 mg/g at 31 weeks; n=4). Thus, administration of a subdepressor dose of ACE inhibitor might prevent rather than delay LV remodeling.

Restrainment of the inflammatory process by ACE inhibition may at least partly contribute to the decrease in gene expression and activity of MMPs. However, macrophages are unlikely to be the sole cell type to produce MMPs (Figure 6B). Angiotensin II activates protein kinase C, which is involved in intracellular induction of MMP transcription, and long-term ACE inhibition decreased mRNA levels of MMPs in this study. Thus, the ACE inhibitor–induced downregulation of the transcription of MMPs in other cells might also be responsible for the attenuation of MMP expression and activity.

The long-term ACE inhibition suppressed MMPs, which contribute to the degradation of collagens, but prevented collagen accumulation, one of major causes for HF. The ACE

Figure 4. A. Representative gelatinolytic bands at 72-kDa region (MMP-2) and 92-kDa region (MMP-9). B. Summary data of 72-kDa gelatinase activity. w indicates week. *P<0.05 vs 23wN group, †P<0.05 vs 23wACEI(−) group, ‡P<0.05 vs 26wN group, ¶P<0.05 vs 26wACEI(−) group at same time point. Values are mean±SEM.

Figure 5. Film in situ zymography presented apparent digestion in LV wall in an ACE(−) rat at 23 weeks and further digestion at 26 weeks. In ACE(+) rats at 23 and 26 weeks, gelatinolytic activity was attenuated. Rats of N group at 23 and 26 weeks showed minimal gelatin lysis.

Figure 6. A. Representative immunohistostainings for macrophages at 23 and 26 weeks in rats from N group, ACEI(−) group, and ACEI(+) group. B. Representative immunohistostainings in serial sections for macrophages, MMP-2, and MMP-9 in an ACEI(−) rat at 26 weeks.
inhibition also suppressed gene expression of collagens. In the prevention of systolic HF by ACE inhibition, a decrease in collagen content may be provided by decreased collagen production even in association with MMP suppression.

Conclusions
Enhancement of gene expression and activity of MMPs preceded LV dilatation in the hypertensive HF model and progressed with LV geometrical and functional deterioration. ACE inhibition suppressed the preceding activation of MMPs independently of its depressor effect and prevented LV remodeling and dysfunction. Thus, MMPs are likely to trigger LV remodeling and to promote it, and ACE inhibition exerts a direct inhibitory effect on MMPs, leading to the prevention of LV remodeling and dysfunction.

Acknowledgments
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References
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