Role of Osteopontin in Cardiac Fibrosis and Remodeling in Angiotensin II–Induced Cardiac Hypertrophy

Yutaka Matsui, Nan Jia, Hiroshi Okamoto, Shigeyuki Kon, Hisao Onozuka, Masatoshi Akino, Lizhi Liu, Junko Morimoto, Susan R. Rittling, David Denhardt, Akira Kitabatake, Toshimitsu Uede

Abstract—Osteopontin (OPN) is upregulated in several experimental models of cardiac fibrosis and remodeling. However, its direct effects remain unclear. We examined the hypothesis that OPN is important for the development of cardiac fibrosis and remodeling. Moreover, we examined whether the inhibitory effect of eplerenone (Ep), a novel aldosterone receptor antagonist, was mediated through the inhibition of OPN expression against cardiac fibrosis and remodeling. Wild-type (WT) and OPN-deficient mice were treated with angiotensin II (Ang II) for 4 weeks. WT mice receiving Ang II were divided into 2 groups: a control group and an Ep treatment group. Ang II treatment significantly elevated blood pressure and caused cardiac hypertrophy and fibrosis in WT mice. Ep treatment and OPN deficiency could reduce the Ang II–induced elevation of blood pressure and ameliorate the development of cardiac fibrosis, whereas Ep-only treatment abolished the development of cardiac hypertrophy. Most compelling, the reduction of cardiac fibrosis led to an impairment of cardiac systolic function and subsequent left ventricular dilatation in Ang II–treated OPN-deficient mice. These results suggest that OPN has a pivotal role in the development of Ang II–induced cardiac fibrosis and remodeling. Moreover, the effect of Ep on the prevention of cardiac fibrosis, but not cardiac hypertrophy, might be partially mediated through the inhibition of OPN expression. (Hypertension. 2004;43:1195-1201.)

Key Words: extracellular matrix ■ fibrosis ■ hypertrophy ■ remodeling ■ aldosterone ■ mineralocorticoids

Many studies have demonstrated important roles of angiotensin II (Ang II) in the cardiovascular system. Ang II has been implicated in the development of cardiomyocyte hypertrophy and cardiac fibrosis. Myocardial fibrosis is a pathological feature associated with cardiac hypertrophy, and circulating Ang II and aldosterone (Ald) are involved in fibrosis and resultant heterogeneity in tissue structure.1 Progressive cardiac fibrosis contributes to an increase in cardiac muscle stiffness, leading to cardiac dysfunction.2

Osteopontin (OPN) is reported to be involved in the process of Ang II–induced fibrosis.3 Furthermore, OPN can interact with various extracellular matrices, including fibronectin and collagen, suggesting its possible role in matrix organization and stability.4 Recently, it was shown that OPN expression in heart was associated with the development of heart failure.5 Moreover, in a murine model of myocardial infarction, OPN deficiency caused exaggeration of left ventricular (LV) dilation and reduction of collagen deposition compared with wild-type (WT) mice.6 These results suggest that OPN has a pivotal role in cardiac fibrosis and cardiac remodeling.

More recently, an important link was suggested in Ang II–induced cardiac fibrosis between OPN and Ald. Ang II-induced inflammatory damage in coronary arteries and OPN expression, and eplerenone (Ep), a novel Ald receptor antagonist, could inhibit the OPN expression and ameliorate the Ang II–induced inflammatory damage to coronary arteries.7 These results suggested that (1) OPN-mediated vascular inflammation might be part of the mechanism by which the renin-angiotensin-aldosterone system participates in the development of cardiac fibrosis and (2) the effect of Ep on the inhibition of vascular inflammation might be modulated by suppressing OPN expression.

To investigate whether OPN plays a pivotal role in cardiac fibrosis and remodeling, we treated OPN-deficient (OPN−/−) mice with Ang II and compared them with WT mice treated with Ang II alone or with Ang II and Ep. Herein, we report the role of OPN and the relationship between OPN and Ald in cardiac fibrosis and remodeling.

Materials and Methods

Mice

OPN−/− mice were generated as described previously.8 Eight-week-old male OPN−/− mice (n=42) backcrossed to C57BL/6 8 generations and age-matched C57BL/6 male WT mice (n=64) were used.

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1195
**TABLE 1. Body Weight, Systolic BP, HR, and LVW/BW**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=15)</th>
<th>WT/All (n=16)</th>
<th>WT/All/Ep (n=20)</th>
<th>OPN−/− (n=6)</th>
<th>OPN−/−/All (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>25.0±0.3</td>
<td>21.8±0.4†</td>
<td>19.8±0.4††</td>
<td>24.0±0.5</td>
<td>23.0±0.5*</td>
</tr>
<tr>
<td>BP, mm Hg</td>
<td>108.7±0.3</td>
<td>100.8±2.1†</td>
<td>155.5±1.6††</td>
<td>106.4±3.6</td>
<td>162.3±4.9§§</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>445.2±2.6</td>
<td>516.9±4.7†‡</td>
<td>456.2±10.2‡‡</td>
<td>640.0±21.9†</td>
<td>613.9±27.2††</td>
</tr>
<tr>
<td>LVW/BW, mg/g</td>
<td>3.4±0.1</td>
<td>4.5±0.1†‡</td>
<td>3.8±0.1††</td>
<td>3.5±0.1</td>
<td>4.7±0.2†§§</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P<0.05 vs WT; †P<0.001 vs WT; ‡P<0.05; WT/All vs WT/All/Ep or OPN−/−/All; §P<0.05; OPN−/− vs OPN−/−/All.

All animal protocols were approved by the Institutional Animal Care and Use Committee.

**Chronic Administration of Pressor Dose of Ang II**

An osmotic minipump (model 2004, Alza) was implanted subcutaneously into each mouse. Pressor doses of Ang II (2 μg/kg per minute) and saline were administered for 4 weeks. WT and OPN−/− mice (OPN−/−/All) were treated with Ang II. WT mice receiving Ang II were divided into 2 groups: a control group (WT/All) and an Ep treatment group (WT/All/Ep). Ep treatment was done as described previously.

**Blood Pressure and Heart Rate**

Systolic blood pressure (SBP) and heart rate (HR) measurements were done using a tail cuff system (Visitech Systems) at the end of the study. A minimum of 5 preliminary cycles was performed before collecting 10 measurements for each mouse.

**Echocardiographic Analysis**

Transesophageal echocardiography was performed at the end of the study. The expanded method can be found in an online supplement available at http://www.hypertensionaha.org. Isovolumic relaxation time (IRT) was measured to estimate the cardiac diastolic function and was corrected by each RR interval time to compensate for the HR variance, shown as c-IRT.

**Histological Analysis**

Histological analysis was performed using the method provided in the online supplement.

**Reverse Transcription–Polymerase Chain Reaction**

Reverse transcription–polymerase chain reaction (RT-PCR) was performed. The expanded method is provided in the online supplement.

**Immunohistochemistry**

Immunohistochemistry was performed. The expanded method is provided in the online supplement.

**Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End-Labeling Staining**

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was performed. The expanded method is provided in the online supplement.

**Statistical Analyses**

All results are expressed as mean±SEM. Multiple comparisons among 3 or more groups were performed by 2-way ANOVA and Fisher exact test for post hoc analyses. A value of P<0.05 was considered statistically significant.

**Results**

**BP and HR**

The effect of treatment on BP and HR is summarized in Table 1. Before treatment, BP did not differ between WT and OPN−/− mice. Ang II treatment elevated BP in both WT and OPN−/− mice. Both OPN deficiency and Ep treatment significantly reduced the Ang II–induced elevation of BP. Interestingly, the HR in OPN−/− mice was significantly high compared with WT mice. Ang II induced a significant increase of HR in WT mice, whereas it induced a mild but significant decrease of HR in OPN−/− mice. The Ang II–induced increase of HR in WT mice was almost completely abolished by Ep treatment.

**Cardiac Hypertrophy**

LV weight/body weight (LVW/BW) is summarized in Table 1. LVW/BW did not differ between WT and OPN−/− mice. Ang II treatment could increase the LVW/BW in both WT and OPN−/− mice. The Ang II–induced increase of LVW/BW was strongly inhibited by Ep treatment. Cardiac myocyte breadth did not differ between WT and OPN−/− mice. Ang II treatment could similarly increase myocyte breadth in both WT and OPN−/− mice. This Ang II–induced increase of myocyte breadth in WT mice was almost completely abolished by Ep treatment (Figure 1).

**Cardiac Fibrosis**

Representative photomicrographs of the heart and coronary arteries are shown in Figures 2 and 3. Both interstitial fibrosis and perivascular fibrosis were significantly increased after Ang II treatment in WT mice. Importantly, OPN deficiency or Ep treatment almost completely abolished the Ang II–induced interstitial fibrosis and perivascular fibrosis. We further examined whether Ep had any inhibitory effect on Ang II-induced perivascular fibrosis in OPN−/− mice. We found that there was no additional inhibition of perivascular fibrosis in OPN−/− mice by Ep treatment (OPN−/−/All 0.86%±0.11%; OPN−/−/All/Ep 0.83%±0.34%). These results strongly suggested that the effect of Ep on the prevention of perivascular fibrosis was mainly if not totally mediated through the inhibition of OPN expression.

**Cardiac Function and Remodeling**

To investigate cardiac function, we performed echocardiographic examination. Results are shown in Table 2. LV hypertrophy (LVH) judged by posterior wall thickness was...
clearly induced by Ang II treatment in both WT and OPN−/− mice. This Ang II–induced LVH in WT mice was clearly inhibited by Ep treatment. End-diastolic LV diameter and end-systolic LV diameter did not differ among WT, WT/AII, WT/AII/Ep, and OPN−/− mice. Importantly, those 2 parameters were significantly increased in Ang II–treated OPN−/− mice. LV systolic function was measured by percent fractional shortening (%FS). The values for normal %FS in WT mice were consistent with those in previous reports.9,10 This parameter did not differ among WT, WT/AII, and WT/AII/Ep mice. In contrast, this parameter was slightly decreased in OPN−/− mice and further reduced by Ang II treatment, reflecting the impaired LV systolic function in Ang II–treated OPN−/− mice. Moreover, LV diastolic function was evaluated by c-IRT. This parameter was increased in WT mice after Ang II treatment, reflecting the impaired LV diastolic function. On the other hand, it was decreased in both WT/AII/Ep and OPN−/−/AII mice compared with WT/AII mice. This result might reflect the fact that LV diastolic function was improved by Ep treatment or OPN deficiency. Collectively, these results suggested that OPN might play a pivotal role in compensating for Ang II–induced cardiac hypertrophy and remodeling.

Reverse Transcription–Polymerase Chain Reaction
To investigate whether Ang II treatment resulted in the stimulation of signaling cascade, leading to cardiac hypertrophy, OPN−/− mice. Moreover, LV diastolic function was evaluated by c-IRT. This parameter was increased in WT mice after Ang II treatment, reflecting the impaired LV diastolic function. On the other hand, it was decreased in both WT/AII/Ep and OPN−/−/AII mice compared with WT/AII mice. This result might reflect the fact that LV diastolic function was improved by Ep treatment or OPN deficiency. Collectively, these results suggested that OPN might play a pivotal role in compensating for Ang II–induced cardiac hypertrophy and remodeling.

Reverse Transcription–Polymerase Chain Reaction
To investigate whether Ang II treatment resulted in the stimulation of signaling cascade, leading to cardiac hypertrophy.
Ang II treatment in both WT and OPN−/− mice. On the other hand, the SERCA2a and RYR2 mRNA levels were similarly reduced in both WT and OPN−/− mice by Ang II treatment. The BNP mRNA level was elevated in WT mice by Ang II treatment, and Ep treatment only partially reduced this elevation. The BNP mRNA level was significantly increased in OPN−/− mice by Ang II treatment, and this elevation of BNP was significantly higher than that in WT mice.

**Apoptosis**

Ang II treatment increased cardiac cell apoptosis in both WT and OPN−/− mice. However, this increase of Ang II–induced apoptosis in the OPN−/− mice was significantly lower than that in the WT mice. (WT 1 ± 0.5 apoptotic cells/10^5 nuclei; WT/AII 14 ± 13 apoptotic cells/10^5 nuclei [P < 0.05 versus WT]; WT/Ep 5 ± 4 apoptotic cells/10^5 nuclei [P < 0.05 versus WT/AII]; OPN−/− 1 ± 0.8 apoptotic cells/10^5 nuclei; OPN−/−/AII 7 ± 2 apoptotic cells/10^5 nuclei [P < 0.05 versus WT; P < 0.05 versus WT/AII]).

**Immunohistochemistry**

Immunohistochemical staining for OPN, macrophages, and Col-I was performed (Figure I, available online at http://

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**TABLE 2. Echocardiographic Measurements**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n=10)</th>
<th>WT/AII (n=12)</th>
<th>WT/AII/Ep (n=10)</th>
<th>OPN−/− (n=6)</th>
<th>OPN−/−/AII (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PW, mm</td>
<td>0.46 ± 0.05</td>
<td>0.87 ± 0.06†</td>
<td>0.68 ± 0.05‡</td>
<td>0.55 ± 0.04</td>
<td>0.78 ± 0.04§</td>
</tr>
<tr>
<td>LVd, mm</td>
<td>3.14 ± 0.17</td>
<td>3.06 ± 0.14</td>
<td>3.01 ± 0.06</td>
<td>3.18 ± 0.05</td>
<td>3.58 ± 0.15§</td>
</tr>
<tr>
<td>LVds, mm</td>
<td>1.52 ± 0.12</td>
<td>1.48 ± 0.08</td>
<td>1.42 ± 0.06</td>
<td>1.65 ± 0.08</td>
<td>2.09 ± 0.13§</td>
</tr>
<tr>
<td>%FS</td>
<td>51.59 ± 1.32</td>
<td>51.63 ± 1.44</td>
<td>52.82 ± 1.34</td>
<td>48.11 ± 1.25</td>
<td>41.62 ± 1.32§</td>
</tr>
<tr>
<td>c-IRT, ms</td>
<td>0.12 ± 0.02</td>
<td>0.16 ± 0.01†</td>
<td>0.12 ± 0.01‡</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.01‡</td>
</tr>
</tbody>
</table>

PW indicates posterior wall thickness. Values are mean ± SEM. 
*P < 0.05 vs WT; †P < 0.001 vs WT; ‡P < 0.05; WT/WT/AII vs WT/WT/AII/Ep or OPN−/−/All; §P < 0.05; OPN−/− vs OPN−/−/All.
There was no OPN staining in hearts from WT mice (Figure IA). Hearts from Ang II–treated WT mice exhibited OPN staining that was mainly present in the interstitium (Figure IA, arrow). There was no significant expression of OPN in cardiomyocytes (Figure IB). Ep treatment markedly blunted the Ang II-induced OPN staining (Figure IC). As expected, there was no OPN staining in hearts from OPN−/− (Figure ID) and OPN−/−/AII mice (Figure IE). There was little macrophage staining in hearts from WT mice (Figure IF) and OPN−/− mice (Figure II). Macrophages were frequently found associated with the perivascular space (Figure IG, arrow) in hearts from WT/AII mice. On the other hand, this staining was significantly decreased in both WT/AII/Ep (Figure IH) and OP−/−/AII mice (Figure IJ). There was little Col-I staining in hearts from WT mice (Figure IK) and OPN−/− mice (Figure IN). Col-I staining was increased in the WT mouse heart by Ang II treatment (Figure IL, arrow). This staining was clearly abrogated in both WT/AII/Ep (Figure IM) and OP−/−/AII (Figure IO) mice.

**Discussion**

In this study, we demonstrated that (1) Ang II treatment clearly induced the development of hypertension, cardiac hypertrophy, and cardiac fibrosis in WT mice; (2) OPN deficiency could abolish the development of Ang II–induced cardiac fibrosis but not cardiac hypertrophy; (3) conversely, Ep treatment could abolish the development of both cardiac hypertrophy and cardiac fibrosis; and (4) Ang II–treated WT
mice developed prominent concentric cardiac hypertrophy and diastolic dysfunction. However, the systolic function was preserved in those mice. Ep treatment significantly prevented the progression of both concentric cardiac hypertrophy and diastolic dysfunction. However, OPN deficiency led to significant systolic dysfunction and increased LV dilatation after Ang II treatment. These results suggest that OPN has a pivotal role in the development of Ang II–induced cardiac fibrosis and remodeling. Moreover, the effect of Ep on the prevention of fibrosis but not cardiac hypertrophy might partially be mediated through the inhibition of OPN expression.

**OPN and Cardiac Remodeling**

Increasing evidence suggests that OPN has a pivotal role in the progression of cardiac fibrosis. It has been reported that Ang II upregulates OPN mRNA expression in cardiac fibroblasts and that an anti-OPN antibody completely blocks the mitogenic effect of Ang II on rat cardiac fibroblasts. These findings suggest that OPN may be an important mediator of Ang II–induced cardiac fibrosis. Moreover, it was shown that the collagen accumulation in the heart after acute myocardial infarction (AMI) was markedly decreased in OPN−/− mice. These results are consistent with our observations that OPN deficiency markedly prevented the progression of cardiac fibrosis induced by Ang II treatment. Furthermore, the underlying mechanism by which OPN regulates cardiac fibrosis was investigated previously. The attenuation of cardiac fibrosis by OPN deficiency might be attributable to decreased macrophage/monocyte accumulation. This regard, we demonstrated that Ang II–induced macrophage accumulation and cardiac fibrosis in WT mice were almost completely abrogated by the OPN deficiency.

It was shown that OPN expression was enhanced in cardiac hypertrophy and heart failure. However, it is not known whether the elevated OPN expression is a cause or result of cardiac hypertrophy or subsequent remodeling. In this study, we demonstrated that OPN deficiency did not inhibit the progression of cardiac hypertrophy, but rather, led to significant systolic dysfunction and increased LV dilatation after Ang II treatment. These results suggest that OPN played an important role in compensation of Ang II–induced cardiac hypertrophy and remodeling. The precise mechanism by which OPN deficiency affects cardiac remodeling after Ang II treatment has yet to be elucidated. We investigated some possibilities in an attempt to clarify the mechanism causing cardiac systolic dysfunction after Ang II treatment. First, we studied the role of OPN in the production of iNOS because OPN could suppress the cytokine-induced expression of iNOS in cardiac myocytes. However, the iNOS mRNA expression in heart did not differ between WT/AII and OPN−/−/AII mice (Figure 5). Second, we examined the mRNA expression of calcium handling–related protein, SERCA2a, and RYR2 because it was reported that OPN was related to calmodulin–dependent reduction in cytosolic Ca2+ in osteoclasts. However, we found that their expression did not differ between WT/AII and OPN−/−/AII mice (Figure 5). Moreover, we examined cardiac cell apoptosis by TUNEL staining because it was reported that OPN was related to apoptosis. The increase in apoptosis after Ang II treatment was significantly lower in OPN−/− mice than in WT mice. These results suggested that the expression of iNOS, calcium handling–related protein, and cardiac cell apoptosis were not major factors causing cardiac dysfunction and cardiac dilatation in Ang II–treated OPN−/− mice. OPN can interact with collagen, suggesting its possible role in matrix organization and stability. Thus, we hypothesized that dynamic synthesis and breakdown of extracellular matrix (ECM) proteins might play an important role in myocardial remodeling of Ang II–treated OPN−/− mice.

It is reasonable that OPN deficiency could affect myocardial remodeling through the regulation of interstitial ECM and matrix metalloproteinase (MMP) organization but not myocyte function because we observed increased OPN expression only in the interstitium but not in the cardiomyocytes after Ang II infusion. Singh et al reported that OPN−/− mice had greater LV chamber dilatation and decreased collagen accumulation after AMI. More recently, Sam et al reported that OPN−/− mice exhibited increased LV dilatation and reduced fibrosis after Ald treatment. These results are consistent with our finding that the overall cardiac remodeling was impaired despite the decrease of fibrosis and collagen accumulation in OPN−/− mice. They speculated that the major mechanism responsible for chamber dilation after AMI or Ald treatment in OPN−/− mice was a decrease in appropriate interstitial collagen deposition, which led to the side-to-side slippage of myocytes. We also think that side-to-side slippage is a possibly related phenomenon attributable to the loss of beneficial fibrosis, which was first described by Olivetti et al, and is the preferred mechanistic explication in our study. We hypothesized that the loss of an appropriate increase in fibrosis in Ang II–treated OPN−/− mice led to side-to-side slippage of cardiac myocytes, cardiac systolic dysfunction, and cardiac dilatation. However, this hypothesis is still speculative, and further evidence is necessary to support it.

Other factors are also possible because the decreased number of fibroblasts located close to myocytes in general might hamper myocyte function by influencing humoral factors in the interaction of fibroblasts and myocytes. Recently, Xie et al demonstrated that OPN inhibits activation of MMP-2 and MMP-9 by interleukin-1 in cardiac fibroblasts. We think that it is possible that a shift in the cardiac MMP–activity balance toward more activity is involved in both decreased fibrosis and decreased myocyte function. Moreover, it has been shown that myocyte responses are influenced by β-integrins and the ECM. Because OPN is reported to bind to integrin receptors on the cell surface, the last possibility is that OPN directly affects the function of cardiomyocytes through integrins.

**OPN and Ep**

Consistent with a previous report, we found that Ep could abolish the development of cardiac hypertrophy and cardiac fibrosis in a murine model of Ang II–induced hypertension. Because the effects of OPN deficiency and Ep treatment on the prevention of perivascular fibrosis were equivalent in our model, the effect of Ep on the prevention of cardiac fibrosis
might be mediated through the inhibition of OPN. On the other hand, the effects of OPN deficiency and Ep treatment on the prevention of the progression of cardiac hypertrophy and remodeling were quite different in our model. This suggests that Ald may regulate vascular inflammation and cardiac remodeling through both OPN-dependent and independent pathways in this murine model of Ang II–induced cardiac hypertrophy.

**Perspectives**

These data indicate that increased Ang II–induced OPN expression protects against cardiac systolic dysfunction and LV dilatation by promoting collagen synthesis in the interstitial myocardium and plays an important role in cardiac remodeling in hypertensive heart disease. It is generally thought that an increase of interstitial fibrosis is detrimental to cardiac systolic and diastolic function. However, our results demonstrate that an appropriate increase of collagen deposition in the heart might be part of an important compensatory response.

**Acknowledgments**

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**References**


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ROLE OF OSTEOPONTIN IN CARDIAC FIBROSIS AND REMODELING
IN ANGIOTENSIN II-INDUCED CARDIAC HYPERTROPHY

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RUNNING TITLE: Role of osteopontin in cardiac remodeling
EXPANDED MATERIALS AND METHODS FOR ONLINE DATA SUPPLEMENT

Echocardiographic Analysis

Transthoracic echocardiography was performed at the end of the study using an EUB 8000 echocardiographic instrument (Hitachi-Medico, Tokyo, Japan). Mice were anesthetized with pentobarbiturate (70mg/kg IP). End-diastolic left ventricular internal diameter (LVDd), end-systolic left ventricular internal diameter (LVDs), and left ventricular posterior wall thickness (PW) were measured. Percent fractional shortening (%FS) was calculated as %FS=\[(LVDd-LVDs)/LVDd\]x100 to estimate the cardiac systolic function. Isovolumic relaxation time (IRT) was measured to estimate the cardiac diastolic function.

Histological Analysis

For histological analysis, hearts were fixed with 10% formalin by perfusion fixation. Fixed hearts were embedded in paraffin, sectioned at 4µm thickness, and stained with hematoxylin-eosin for overall morphology, or Masson’s trichrome method for fibrosis. Myocyte breadth was measured from sections stained with hematoxylin-eosin, and suitable cross sections were defined as having nearly circular capillary profiles and nuclei. For measurement of the myocyte breadth, 100 cells (per animal) from the left ventricular lateral-mid free wall (including epicardial and endocardial portions) were randomly chosen and analyzed. The collagen fraction was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the myocyte area in the entire visual field of the
section. The area of perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. Approximately 40 arterial cross sections were examined in each heart.

**RT-PCR Analysis**

Reverse transcription–polymerase chain reaction (RT-PCR) with selected primers was used for amplification of atrial natriuretic factor (ANF), collagen-I (Col-I), OPN, inducible nitric oxide synthase (iNOS), SERCA2a, ryanodine receptor 2 (RYR2), brain natriuretic peptide (BNP), and GAPDH mRNA.

ANF primers used were 5'-CTCTGAGAGACGGCAGTGCT-3' (forward) and 5'-ACGGAGAGGGTGAGACGTAT-3' (reverse).

Col-I primers used were 5'-AAACCCGAGGTATGCTTGATCTGTA-3' (forward) and 5'-GTCCCTCGACTCCTACATCTTCTGA-3' (reverse).

OPN primers used were 5'-ATGAGATTGGCAGTGATTTGCTT-3' (forward) and 5'-TTAGTTGACCTGACCAAGATGCACCT-3' (reverse).

iNOS primers used were 5'-ATGGACCAGTATAAGGCAAGC-3' (forward) and 5'-GCTCTGGATGAGCCTATATTG-3' (reverse).

SERCA2a primers used were 5'-GGCTTTTACAGGGCGAGAGT-3' (forward) and 5'-ACCAGATTGACCGAGACTG-3' (reverse).

RYR2 primers used were 5'-GTATGTGGCGTGCCGTGCTG-3' (forward) and 5'-TAGTTGGCCAGGTATGTTC-3' (reverse).

BNP primers used were 5'-ATGGATCTCCTGAAGGTGCT-3' (forward) and
5'-AAGAGGGCAGATCTATCGGA-3' (reverse).

GAPDH primers used were 5'-ATGTTCCAGTATGACTCCACTCAG-3' (forward) and 5'-GAAGACACCAGTAGACTCCACGACA-3' (reverse).

ANF, Col-I, OPN, iNOS, SERCA-2a, RYR2, BNP, and GAPDH sequences were amplified in a thermal cycler (Perkin-Elmer) for optimal numbers of cycles. The quality of RNA preparation and cDNA synthesis was verified by amplifying DNA coding GAPDH, a housekeeping protein, under the same conditions. RT-PCR products were visualized on 2% agarose gels with ethidium bromide. Signals were digitized and evaluated with an optical scanner (GT-9500, Seiko) with density measured using the image analysis program NIH IMAGE.

**Immunohistochemistry**

Monoclonal Abs, OPN 2.2 reacting against mouse OPN (IBL, Gumma, Japan), and F4/80 reacting against macrophages (Serotec, Tokyo, Japan), and a polyclonal Ab reacting against Col-I (CALBIOCHEM, CA, USA), were used in this study. The sections were stained with OPN2.2 and F4/80 followed by biotin-conjugated goat anti-rat IgG, or anti-Col-I Ab followed by biotin-conjugated goat anti-rabbit IgG, and streptavidin-biotin peroxidase complex (Histofine kit; Nichirei, Tokyo, Japan), and counterstained with hematoxylin.

**TUNEL Staining**

To detect apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining followed by Hoechst 33258 staining was carried out in 4-µm-thick sections.
TUNEL-positive nuclei that appeared within the cardiac myocytes were counted. The total number of nuclei per unit area of the heart was estimated by counting the number of Hoechst-positive nuclei under ultraviolet illumination. The number of apoptotic cardiac myocyte nuclei in 15 fields was averaged, and the data were calculated as the percentage of apoptotic myocyte nuclei/total number of nuclei.
FIGURE LEGENDS FOR ONLINE DATA SUPPLEMENT

Figure I. Immunohistochemical localization of OPN (A-E), macrophages (F-J), and Col-I (K-O) in left ventricles of mice. The heart of WT (A, F, K), WT/AII (B, G, L), WT/AII/Ep (C, H, M), OPN⁻/⁻ (D, I, N), and OPN⁻/⁻/AII (E, J, O) mice were stained, respectively (original magnification ×400).