Heparin Cofactor II Protects Against Angiotensin II–Induced Cardiac Remodeling Via Attenuation of Oxidative Stress in Mice

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Abstract—Heparin cofactor II (HCII), a serine protease inhibitor, inhibits tissue thrombin action after binding with dermatan sulfate proteoglycans in the extracellular matrix of the vascular system. We previously reported that heterozygous HCII-deficient (HCII+/−) humans and mice demonstrate acceleration of vascular remodeling, including atherosclerosis. However, the action of HCII on cardiac remodeling never has been determined. HCII+/− and HCII+/− mice at age 25 weeks were infused with angiotensin II (Ang II; 2.0 mg/kg/d) for 2 weeks by an osmotic mini-pump. Echocardiography revealed acceleration of cardiac concentric remodeling in HCII+/− mice and larger left atrial volume in HCII+/− mice than in HCII+/+ mice. Histopathologic studies showed more prominent interstitial fibrosis in both the left atrium and left ventricle in HCII+/− mice than in HCII+/+ mice. Daily urinary excretion of 8-hydroxy-2′-deoxyguanosine, a parameter of oxidative stress, and dihydroethidium-positive spots, indicating superoxide production in the myocardium, were markedly increased in Ang II-treated HCII+/− mice compared to those in HCII+/+ mice. Cardiac gene expression levels of atrial natriuretic peptides and brain natriuretic peptides, members of the natriuretic peptide family, Nox 4, Rac-1, and p67phox as components of NAD(P)H oxidase, and transforming growth factor-β1 and procollagen III were more augmented in HCII+/− mice than in HCII+/+ mice. However, administration of human HCII protein attenuated all of those abnormalities in Ang II-treated HCII+/− mice. Moreover, human HCII protein supplementation almost abolished cardiac fibrosis in Ang II-treated HCII+/− mice. The results indicate that HCII has a protective role against Ang II–induced cardiac remodeling through suppression of the NAD(P)H oxidase–transforming growth factor-β1 pathway. (Hypertension. 2010;56:430-436.)

Key Words: angiotensin II ■ cardiac remodeling ■ heparin cofactor II ■ oxidative stress

Thrombin not only acts as a coagulation key enzyme but also is involved in tissue repair and remodeling, embryogenesis, angiogenesis, and development of atherosclerosis.1,2 These biological functions of thrombin appear to be mediated by specific thrombin receptors, particularly protease-activated receptor-1 (PAR-1).1,2 Because it has been reported that PAR-1 is expressed in cardiomyocytes and cardiac fibroblasts3,4 and that PAR-1 contributes to cardiac remodeling and hypertrophy,5 there is a possibility that modulation of the thrombin–PAR-1 axis affects cardiac remodeling. Thrombin is inactivated by 2 major coagulation modulators: antithrombin and heparin cofactor II (HCII).6,7 In circulating blood of the intravascular lumen, antithrombin binding to heparan sulfate inhibits thrombin action, whereas in the subendothelium HCII binding to dermatan sulfate inhibits thrombin action. Therefore, we hypothesized that HCII exerts antivascular remodeling effects through inactivation of the thrombin–PAR-1 axis. We previously reported that high-plasma HCII activity is associated with reduced incidence of in-stent restenosis after percutaneous coronary intervention and that plasma HCII activity was negatively correlated with carotid maximum plaque thickness and prevalence of peripheral arterial disease in humans.8–11 In experimental animal studies, we and Tollefsen et al12,13 demonstrated that HCII-deficient mice manifest prominent intimal hyperplasia with increased cellular proliferation after tube cuff and wire vascular injury. Conversely, the intimal hyperplasia in HCII+/− mice with vascular injury was abrogated by human HCII supplementation.12 These results indicated that HCII plays a protective role against the progression of vascular remodeling. Because it has been recognized that development of vascular remodeling, including atherosclero-
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Materials and Methods

Animal Preparations

We used HCII+/+ male mice and HCII−/− male mice (HCII+/+; The University of Tokushima, Graduate School of Health Biosciences, Tokushima, Japan) that we previously generated at 25 weeks of age.12 The mice underwent sham operation or were infused with Ang II (WAKO) at a rate of 2.0 mg/kg per day for 2 weeks by an osmotic mini-pump (Alzet model 1002; Alza) as previously described.16–18 One group of Ang II-treated HCII+/+ mice was administered human purified HCII protein 3 times per week for 2 weeks as previously reported.12 And 1 group of Ang II-treated HCII−/− mice was also administered human purified HCII protein 3 times per week for 2 weeks. All experimental procedures were performed in accordance with the guidelines and approval from the Animal Research Committee of The University of Tokushima Graduate School. We performed the following experimental procedures: echocardiographic analysis, histological analysis and immunohistochemistry, analysis of urinary excretion of 8-hydroxy-2-deoxyguanosine, superoxide detection in myocardial tissues, evaluation of plasma superoxide dismutase activity, quantitative real-time polymerase chain reaction analysis, and Western blot analysis as detailed in the online data supplement (please see http://hyper.ahajournals.org).

Statistical Analysis

All data are expressed as means±SEM. For comparisons among groups, statistical significance was assessed using a 1-way analysis of variance and the significance of each difference was determined by post hoc testing using the Tukey-Kramer method. Statistical significance was considered at P<0.05.

Results

Prominent Concentric Cardiac Remodeling in Ang II-Treated HCII+/+ Mice

Although systolic blood pressure levels were elevated to ≈40 mm Hg beyond baseline blood pressure by Ang II infusion, HCII deficiency did not affect levels of systolic blood pressure and heart rate during the experimental period, regardless of Ang II infusion in the mice (Figure 1A–C). There was no difference in fractional shortening percentage among the groups regardless of Ang II infusion (Figure 1A–C). Augmented relative wall thickness, but not left ventricular mass index, indicating prominent concentric cardiac remodeling was observed in Ang II-treated HCII+/+ mice compared to that in Ang II-treated HCII−/− mice (Figure 2A–C and Figure 3A). When human HCII protein was administered to HCII−/− mice, the Ang II–induced alteration was attenuated (Figure 2A, B and Figure 3A).

Increased Left Atrial Volume in Ang II-Treated HCII+/+ Mice

Echocardiographic analysis showed that Ang II stimulation increased left atrial volume in HCII+/+ mice as well as in HCII−/− mice (Figure 2E, F). The enlarged left atrial volume was much greater in HCII+/− mice than in HCII−/− mice (Figure 2E, F). However, HCII supplementation attenuated left atrial volume enlargement in Ang II-treated HCII+/− mice to almost the same level as that in Ang II-treated HCII+/+ mice (Figure 2E, F). These observations were consistent with macroscopic findings as shown in Figure 3A.

Exacerbation of Cardiac Fibrosis in Ang II-Treated HCII+/+ Mice

There were no morphological differences in left ventricular mass, left atrial tissues, and ventricular tissues between HCII+/+ mice and HCII−/− mice without Ang II infusion (Figure 3A). Ang II stimulation caused not only prominent...
These unfortunate changes in hearts of HCII mice (Figure 4A). Human HCII protein treatment significantly attenuated the Ang II–induced augmentation of cardiac superoxide production and elevation of urinary 8-hydroxy-2’-deoxyguanosine excretion in HCII mice (Figure 4A). Although we also evaluated plasma superoxide dismutase (SOD) activity, indicating antioxidant capacity, there was no significant difference among the mice groups regardless of Ang II infusion (Figure 4B).

**Accelerated Gene Expression of Natriuretic Peptide and Procollagen III But Not PAR-1 in Cardiac Tissue of Ang II-Treated HCII** 

It has been well-known that natriuretic peptides, including atrial natriuretic peptides and brain natriuretic peptides, and procollagen III and PAR-1 are upregulated during cardiac remodeling, leading to cardiac failure. Therefore, we evaluated cardiac expression of those genes in the present study. There was no significant difference in mRNA levels of those genes in HCII mice compared to those in HCII mice (Figure 3A–C). These unfortunate changes in hearts of HCII mice were also restored by human HCII protein supplementation (Figure 3A–C).

**Augmented Oxidative Stress in Ang II-Treated HCII** 

To evaluate Ang II–induced superoxide production, we analyzed dihydroethidium (DHE) staining of the atria and ventricles of the mice by fluorescence microscopy. Ang II stimulation increased cardiac superoxide production to a greater extent in HCII mice than in HCII mice (Figure 4C–E). Next, we estimated urinary excretion of 8-hydroxy-2’-deoxyguanosine as an oxidative stress marker. In parallel with the results of cardiac DHE staining, Ang II treatment caused a more notable increase of urinary 8-hydroxy-2’-deoxyguanosine excretion in HCII mice than in HCII mice (Figure 4A). Human HCII protein treatment significantly attenuated the Ang II–induced augmentation of cardiac superoxide production and elevation of urinary 8-hydroxy-2’-deoxyguanosine excretion in HCII mice (Figure 4A).
NAD(P)H oxidase system,\(^{19,20}\) we examined the mRNA levels of NAD(P)H oxidase components, including Nox subunits, p22\(^{\text{phox}}\) for membrane subunits, p67\(^{\text{phox}}\) and p47\(^{\text{phox}}\) for cytosol subunits, and the small GTP-binding protein Rac-1 in the heart. Although no difference was observed between the levels of NAD(P)H oxidase expression in the mice without Ang II infusion, Ang II-treated HCII\(^{+/+}\) mice showed higher mRNA expression levels of Nox 4, p67\(^ {\text{phox}},\) and Rac-1, but not p22\(^ {\text{phox}}\) and p47\(^ {\text{phox}},\) than those in Ang II-treated HCII\(^{+-}\) mice (Figure 5). Ang II plays a pivotal role in tissue fibrosis, partly through increasing production of transforming growth factor (TGF)-\(\beta 1\), which is a potent accelerator in extracellular matrix remodeling. Therefore, we evaluated expression levels of TGF-\(\beta 1\) mRNA and protein in the mice with and without Ang II stimulation. In this study, Ang II-treated HCII\(^{+/+}\) mice showed prominent TGF-\(\beta 1\) mRNA and protein expression compared with that in Ang II-treated HCII\(^{+-}\) mice (Figures 5, 6A). Moreover, immuno-}

![Figure 3](image-url)

**Figure 3.** Pathological findings of hearts in HCII\(^{+/+}\) and HCII\(^{+-}\) mice with and without Ang II stimulation. A, Representative findings of Masson-Trichrom-stained cardiac tissues. Upper panels show macroscopic findings of transverse sections of the left ventricular papillary muscle level. Middle panels show microscopic findings of the left atrial myocardium. Lower panels show microscopic findings of the left ventricular myocardium. B and C, Quantitative results of left atrial and ventricular fibrosis. Values are mean±SE. *\(P<0.01\) vs control of the same genotype; #\(P<0.05\) vs Ang II-infused HCII\(^{+/+}\) mice; $\(P<0.05\) vs Ang II-infused HCII\(^{-/-}\) mice, n=20 to 26 in each group. HCII\(^{+/+}\) mice without Ang II (20), HCII\(^{-/-}\) mice without Ang II (22), HCII\(^{+/+}\) mice with Ang II (22), HCII\(^{-/-}\) mice with Ang II (26), HCII\(^{-/-}\) mice with Ang II, and h-HCII (24).

![Figure 4](image-url)

**Figure 4.** Evaluation of oxidative stress and antioxidant factor in HCII\(^{+/+}\) and HCII\(^{-/-}\) mice with and without Ang II stimulation. A, Quantification of daily urinary excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG). B, Plasma SOD activity levels, n=8 in each group. C, Representative findings of superoxide detection using DHE staining in the left atrial and ventricular myocardium. D and E, Quantification of fluorescent intensity of DHE- positive area. Values are expressed as mean±SE. *\(P<0.01\) vs control of the same genotype; #\(P<0.05\) vs Ang II-infused HCII\(^{+/+}\) mice; $\(P<0.05\) vs Ang II-infused HCII\(^{-/-}\) mice, n=20 to 26 in each group except plasma SOD activity measurement. HCII\(^{+/+}\) mice without Ang II (20), HCII\(^{-/-}\) mice without Ang II (22), HCII\(^{+/+}\) mice with Ang II (22), HCII\(^{-/-}\) mice with Ang II (26), HCII\(^{-/-}\) mice with Ang II, and h-HCII (24).

![Figure 5](image-url)

**Figure 6.** Immunohistochemistry of left ventricular tissues showed a larger number of TGF-\(\beta 1\)-stained spots in cardiomyocytes and interstitial areas with fibrotic change in Ang II-treated HCII\(^{+/+}\) mice than in Ang II-treated HCII\(^{-/-}\) mice (Figure 6B). Conversely, human HCII protein administration abolished the enhancement of the cardiac NAD(P)H oxidase–TGF-\(\beta 1\) pathway in Ang II-infused HCII\(^{-/-}\) mice (Figures 5, 6A, B).

**Excess of Human HCII Protein Attenuates Cardiac Fibrosis Even in Ang II-Infused HCII\(^{+/+}\) Mice**

To clarify the dose-dependency of the cardiac-protective action of HCII against Ang II excess, we quantified fibrosis areas in cardiac tissues of Ang II-infused HCII\(^{+/+}\) mice with and without human HCII supplementation. As shown in
HCII supplementation reduced the amount of oxidative stressinduced NAD(P)H oxidase stimulation. Because previous studies showed that thrombin activates NAD(P)H oxidase, leading to increased oxidative stress in vascular endothelial cells and vascular smooth muscle cells, HCII may contribute to reducing cardiac superoxide production via inhibition of thrombin-induced NAD(P)H oxidase stimulation.

TGF-β1 is a crucial mediator of cardiac adaptation to hemodynamic overload and is closely associated with the pathogenesis of cardiac remodeling such as cardiac hypertrophy and heart failure. TGF-β1 has been shown to be a powerful initiator of cellular hypertrophy and interstitial fibrosis in the heart and to be involved in NAD(P)H oxidase activation. These findings concerning the interplay between NAD(P)H oxidase and TGF-β1 are consistent with the molecular phenotypes of accelerated cardiac remodeling in Ang II-treated HCII−/− mice.

Recent studies have shown that cardiac fibrosis is a process characterized by massive remodeling of the myocardial extracellular matrix and subsequent substitution of functional tissue by inelastic fibrotic tissue leading to failing heart. Vanhoutte et al demonstrated that syndecan-1, which is a transmembrane (type I) heparan sulfate proteoglycan and a member of the syndecan proteoglycan family, plays a pivotal role in protection against exaggerated inflammation and adverse infarct healing after myocardial infarction in mice.
Moreover, Hong et al. showed that supplementation with dermatan sulfate, a glycosaminoglycan, improved cardiac function and maintained viability of cardiac myocytes with complement activation. Based on these observations and the fact that HCII has the ability to inhibit thrombin action by formation of a bimolecular complex with dermatan sulfate, the bimolecular complex with HCII and dermatan sulfate might cooperatively have beneficial effects in the heart against cardiac stress, including ischemia and Ang II excess.

Because the enhanced expression of PAR-1, in turn, is expected to further promote cardiac remodeling, we speculated that the organ-protective action of HCII is exerted on cardiomyocytes in which the thrombin–PAR-1 axis is activated to stimulate cardiomyoelastic remodeling. However, in the present study, Ang II-treated HCII−/− mice showed no difference in mRNA levels of cardiac PAR-1 (Figure 5) compared to those in Ang II-treated HCII+/+ mice. Moreover, it is unlikely that inactivation of the thrombin–PAR-1 axis by HCII is the only mechanism for reduction of oxidative stress against Ang II infusion. Therefore, there is a possibility that HCII independently and directly modulates expression of NAD(P)H oxidase components in cardiac tissue conditioned with an excess of Ang II (Figure 6D). Further examinations are needed to clarify this issue.

All of these observations in the present study were further corroborated by results of a clinical study about cardiac remodeling and plasma HCII activities (T. Ise, K. Aihara, Y. Sumitomo-Ueda, S. Yoshida, Y. Ikeda, S. Yagi, T. Iwase, H. Yamada, M. Akaite, M. Sata, T. Matsumoto, unpublished observations, 2010). In that study, we investigated the relationship between plasma HCII activity and cardiac remodeling surrogate markers measured by echocardiography, and we found that plasma HCII activity is independently and inversely associated with the development of cardiac remodeling, including cardiac concentric change, left atrial enlargement, and left ventricular diastolic dysfunction determined by increased E-to-Ea (peak E velocity to early diastolic mitral annulus velocity) ratio. Therefore, the observations in our animal model might be applicable to human hypertensive heart disease in HCII deficiency.

**Perspectives**

HCII counteracts the development of cardiac remodeling, including cardiac concentric changes, left atrium enlargement, and cardiac fibrosis associated with regulating oxidative stress. These results indicate that treatment with HCII might be a novel and valuable therapeutic approach to prevent cardiac remodeling and atherosclerosis.
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Disclosures
None.

References
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In the Hypertension article by Sumitomo-Ueda et al (Sumitomo-Ueda Y, Aihara K, Ise T, Yoshida S, Ikeda Y, Uemoto R, Yagi S, Iwase T, Ishikawa K, Hirata Y, Akaike M, Sata M, Kato S, Matsumoto T. Heparin Cofactor II Protects Against Angiotensin II–Induced Cardiac Remodeling Via Attenuation of Oxidative Stress in Mice. Hypertension. 2010;56:430–436), changes have been made to the Results section, second paragraph, second sentence. The types of mice have been modified. The sentence now reads as follows: “The enlarged left atrial volume was much greater in HCII<sup>−/−</sup> mice than in HCII<sup>+/+</sup> mice (Figure 2E, F).”

The authors regret the error.

This correction has been made to the current online version of the article, which is available at http://hyper.ahajournals.org/cgi/content/full/56/3/430.
Online Supplement

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Echocardiographic Analysis

Transthoracic echocardiography was performed using a 15-MHz imaging transducer (Aplio 80; Toshiba Medical Systems Co. Ltd., Tochigi, Japan). Mice from each group were anesthetized by peritoneal injection with 20 mg/kg of 2.5% pentobarbital and 8 mg/kg of 2.0% xylazine. The left hemithorax of each mouse was carefully shaved, and M-mode images of the left ventricle (LV) were recorded. Left ventricular anterior wall thickness (AW), left ventricular end-diastolic dimension (LVDd), left ventricular end-systolic dimension (LVDs), and left ventricular posterior wall thickness (PW) were measured. All measurements were performed using the leading edge-to-edge convention adopted by the American Society of Echocardiography. Percent fractional shortening and relative wall thickness were calculated. B-mode images of the left atrium (LA) were recorded. LA volume was calculated by the area-length method and biplane-modified Simpson method. The sonographer was well-trained and blinded to the murine genotype in this study and the echocardiographical measurements were repeated 3 times and averaged for each mouse. The coefficients of variance for intra-assays of echocardiographical measurements, including AW (5.22%), PW (3.42%), LVDd (3.87%), LVDs (4.65%), LAV (Area-Length Method: 4.08%, Modified Simpson Method: 4.84%), confirmed the reliability of the study.

Histological Analysis and Immunohistochemistry

Whole hearts were resected and placed in 10% neutral buffered formalin overnight. After fixation, the samples were embedded in paraffin. Sections (3-µm-thick) of the heart were cut and stained with Masson Trichrome staining. The cross-sectional areas of LV myocytes were measured on the mid free wall of the LV from sections. Suitable cross-sectional areas were defined as having nearly circular capillary profiles and nuclei. Approximately 100 cells were counted in each section and the average area was used for analysis. To calculate the ratio of the interstitial fibrosis area in the left ventricular area, excluding perivascular fibrosis, 10 fields of samples were randomly selected from 3 individual sections. Moreover, we measured the perivascular fibrosis area, outer vascular area, and medial area of coronary arteries in each section. The index of perivascular fibrosis was calculated as the ratio of the pericoronary fibrosis area to the outer vascular area. The index of arterial medial thickening was calculated as the ratio of the medial area to the outer vascular area. Measurements of the areas were performed using ImageJ 1.29, a free software program.
Samples of hearts after fixation with paraffin, as described above, were stained using an anti-TGF-β1 rabbit polyclonal antibody (sc-146; Santa Cruz Biotechnology, Inc, USA) at a dilution of 1:50.

**Analysis of the Urinary Excretion of 8-hydroxy-2′-deoxyguanosine**

The formation of reactive oxygen species is a critical event in cardiovascular remodeling because it promotes cell proliferation, hypertrophy, growth arrest, and/or apoptosis and oxidation of LDL-cholesterol. Martinet et al. revealed that 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-OHdG) can be used as a marker of oxidative DNA damage and that it is increased in human atherosclerotic plaques (*Circ Res.* 2001;88:733-739.). Therefore, increased 8-OHdG levels are thought to be an appropriate marker for Ang II-induced DNA damage during cardiovascular remodeling. In this study, the urinary excretion of 8-OHdG was measured using an enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (New 8-OHdG Check ELISA Kit; Japan Institute for the Control of Aging, Nikken SEIL Corporation, Shizuoka, Japan). Mice were housed individually in metabolic cages that provided 24 h free access to tap water and food. We measured the concentration of urinary 8-OHdG to determine the total oxidative stress and corrected those levels by the volume of daily urinary excretion and body weight.

**Analysis of Plasma Superoxide Dismutase Activity**

Before excising the hearts, overnight fasting blood samples were collected in a tube containing heparin. Each blood sample was immediately centrifuged (3,000 × g for 10 min at 4°C) to separate plasma, which was stored at −80°C for later analysis. The activity of superoxide dismutase (SOD) was measured by the modified nitrous acid method.

**Quantitative Real-time PCR**

RNA extraction and RT-PCR were performed. Briefly, total RNA was isolated from heart tissue using TRIzol (Life Technologies Inc., Rockville, MD, USA) and then treated with deoxyribonuclease I (ribonuclease free; Takara Bio Inc., Ohtsu, Japan). Total RNA (1 µg) was used for cDNA synthesis with an ExScript RT Reagent Kit
The PCR mixture contained cDNA equal to 5 ng total RNA, 0.1 nmol/l forward and reverse primer mix, and SYBR Green (Platinum SYBR Green quantitative PCR SuperMix-Uracil-DNA Glycosylase (UDG) with ROX Reference Dye; Invitrogen, Tokyo, Japan). Assays were performed using an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA). Amplification included one stage of 2 min at 95°C followed by 50 cycles of a two-step loop: 10 s at 95°C and 30 s at 60°C. Results were analyzed using SDS 7300 software. Primers for Nox 4, p67phox, and TGF-β1 were obtained from QIAGEN (QuantiTect Primer Assay®, Tokyo, Japan). BNP, β-Actin, PAR-1 and procollagen III were obtained from Takara Bio (Perfect Real-time Primer®, Ohtsu, Shiga, Japan). A customized primer set for ANP was designed: forward; 5′-ATT TCA AGA ACC TGC TAG ACC ACC T-3′ and reverse; 5′-CAG TCT GCT CAC TCA GGG CC-3′. A customized primer set for Rac-1 was designed: forward; 5′-CCA GTG AAT CTG GGC CTA TG-3′ and reverse; 5′-ACA GTG GTG TCG CAC TTC AG-3′. The specificity and efficiency of the primers were confirmed by checking the melting curve and PCR efficiency calculated from the standard curve using adjunctive analyzing software. As the melting curve had a single peak and the PCR efficiency was more than 80% for all of the primers, we considered the PCR primers appropriate for real-time PCR. The mRNA expression levels of ANP, BNP, PAR-1, procollagen III, Nox 4, Rac-1, p67phox, and TGF-β1 were normalized using β-actin as the internal control. Eighteen to 24 independent samples in each group were used for real-time PCR and triplicate results per sample were averaged.

Western Blot Analysis

TGF-β1 protein expression was evaluated using western blot analysis as previously described. We used antibodies against TGFβ-1 (Santa Cruz Biotechnology, Inc., CA, USA) and β-actin (Biolegend, San Diego, CA, USA). For western blot analysis, 100 µg protein extracts from the hearts of HCII+/+ and HCII+/− mice were boiled for 5 min in Laemmli sample buffer and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein extracts were then transferred to a nitrocellulose membrane (Hybond TM-ECL; Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked for 1 h at room temperature with 5% non-fat skinned milk in phosphate-buffered saline/Tween 20.

(Takara Bio, Ohtsu, Shiga, Japan) according to the manufacturer’s instructions. The
(PBS-T). The blots were incubated overnight at 4°C with antibodies for TGF-β1 followed by an incubation for 1 h with anti-rabbit secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence with an ECL reagent (Amersham ECL Plus Western Blotting Detection System; GE Healthcare, Buckinghamshire, UK) treatment and exposure to Hyperfilm-ECL. The intensity of the bands was measured using ImageJ version 1.29.

**Superoxide Detection in Myocardial Tissue**

We evaluated cardiac superoxide production using in situ dihydroethidium (DHE) staining. In brief, hearts were excised from mice, rinsed in physiological saline, and frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Fine Chemical, Tokyo, Japan) until use. Transverse sections (10-µm-thick) were cut with a cryostat and placed on silane-coated glass slides. The sections were incubated with DHE in PBS (10 mmol/l) in a dark, humidified container at room temperature for 30 min. DHE is oxidized upon the reaction of superoxide with ethidium bromide, which binds to DNA in the nucleus and fluoresces red. After placing a cover slip over the heart tissues, the tissues were observed using a laser scanning confocal microscope (Leica TCS-NT, mounted on a Leica DMRB light microscope; Leica, Mannheim, Germany). The excitation wavelength was 488 nm and the emission fluorescence was detected using a 568 nm long-pass filter.