Iron plays an important role in maintaining physiological homeostasis in the body (i.e., enzymatic reactions and oxygen transport). However, excess iron can lead to free radical damage by the Fenton reaction, resulting in tissue damage. During the past decade, iron has been associated with the pathogenesis of some cardiovascular diseases. For instance, iron deposition is related to development of atherosclerosis, and an experimental study showed that an iron-deficient diet reduces atherosclerotic lesions in apolipoprotein-E-deficient mice.1 In addition, a multi-center, randomized, controlled, single-blinded clinical trial showed that cancer risk and cancer-specific and all-cause mortality were lower in the iron reduction (IR) group than in the control group in patients with peripheral arterial disease.2 These results suggest that excess total body iron stores are associated with the occurrence of cancer and longevity in patients with peripheral arterial disease.

Caloric restriction has been shown to extend longevity by retarding the aging process.3 Caloric restriction also reduces blood pressure and dyslipidemia.4 In cardiovascular diseases, several experiments with animal studies have shown that caloric restriction has protective effects against cardiac hypertrophy, salt-induced cardiac remodeling,5 and ischemia-reperfusion injury.5 However, the effects of only IR on cardiovascular disease, particularly salt-induced organ damage, remain largely unknown. In the present study, we investigated the effects of dietary IR on salt-induced cardiovascular pathophysiology and its involved mechanism in Dahl salt-sensitive rats. Our observations indicate that dietary IR has salutary effects on salt-induced hypertension, cardiovascular remodeling, and proteinuria by reducing oxidative stress and by improving impairment of vascular Akt, AMP-activated protein kinase (AMPK), and endothelial nitric oxide synthesis (eNOS) signaling.
Animals

Five-week-old male Dahl salt-sensitive rats (Japan SLC) were fed a control diet (0.3% NaCl) for 1 week. Afterward, rats were randomly assigned to 3 groups and were given a normal salt diet (control; 0.3% NaCl; n = 12), a high-salt (HS) diet (8% NaCl; n = 12), or an HS diet with iron-restricted diet (HS + IR; n = 12) for 12 weeks. Regular rat chow was supplemented with approximately 0.003% of FeC₅H₄O₂·H₂O. Rats of the HS + IR group were given a diet not supplemented with FeC₅H₄O₂·H₂O as previously described.7 In a separate study, 10-week-old HS + IR group rats, which had been fed that diet from 6 weeks of age, were divided into 2 groups and were given a HS + IR diet with plain drinking water (n = 6) or N^ω-nitro-L-arginine methyl ester (l-NAME; Sigma-Aldrich), a specific nitric oxide (NO) synthase inhibitor (0.25 mg/mL in drinking water; n = 6). Rats were maintained on a 12-hour light/dark cycle and had free access to food and water. All our experimental procedures were approved by the Animal Research Committee of Hyogo College of Medicine. An expanded Methods section is available in the online data supplement at http://hyper.ahajournals.org.

Gene Expression Analysis

Total RNA was extracted from the tissue using TRIzol reagent (Invitrogen).8 Real-time polymerase chain reactions were performed using the ABI PRISM 7900 with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems).3 Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Western Blot Analysis

The total protein homogenate from the aorta was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The expression levels of proteins were detected by an enhanced chemiluminescence method. The total protein homogenate from the aorta was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The expression levels of proteins were detected by an enhanced chemiluminescence method.

Histological Analysis

Aorta and kidney tissues were quickly embedded in Tissue-Tek OCT compound (Sakura Finetechical Co.) and were snap frozen in liquid nitrogen. Aortic sections were stained with Masson’s trichrome (MT) and immunohistochemically stained with a primary mouse anti-CD68 antibody (AbD Serotec; dilution 1:1000) and a primary mouse anti-TfR1 antibody (Zymed Laboratories; dilution 1:400). Nonimmune immunoglobulin G of the same species was used as a negative control. Superoxide detection was performed on transverse cross-sections 8-μm thick, incubated with dihydroethidium (DHE; 10 μmol/L, 37°C for 30 minutes; Molecular Probes).

Statistical Analysis

Values are reported as the means ± SEM. Statistical analysis was performed using one-way ANOVA, ANOVA (Kruskal-Wallis test, followed by Mann-Whitney U test) was used for statistical comparisons. Survival rate was assessed by the Kaplan–Meier survival curves. We considered differences to be significant when the probability value was < 0.05.

Results

Effects of Iron Restriction on Physiological Parameters

Body weight decreased significantly in the HS group after 14 weeks of age compared with the other groups, while the HS + IR group did not lose body weight until 18 weeks of age, and the degree of loss was smaller than in the HS group at the same time (Figure 1A); this suggests that IR in the presence of HS diet prevented the onset of cachexia with the development of heart failure. Dietary IR-induced anemia was measured by hemoglobin content (g/dL) in all groups studied, whereas blood hemoglobin was comparable between control and HS groups until 10 weeks of age and began to decrease in the HS group thereafter. Finally, blood hemoglobin was lower in the HS + IR group than in the HS group at 18 weeks of age (Figure 1B). Conversely, HS diet resulted in a progressive increase in systolic blood pressure (SBP) after diet, while SBP did not begin to increase in the
HS+IR group until 14 weeks of age and the increase was smaller than in the HS group (Figure 1C); this indicates that IR in the presence of HS diet inhibited the increase in SBP. During the experimental period, some rats died in the HS group; however, none of rats died in the HS+IR group. Kaplan–Meier analysis showed that survival rate of the IR group (Figure 1D).

**Iron Restriction Reduced Vascular Hypertrophy, Fibrosis, and Inflammation in High Salt-Induced Hypertension**

Since IR rats did not develop hypertension, we evaluated vascular hypertrophy, fibrosis, and inflammation in these groups. MT staining showed vascular hypertrophy and increased fibrotic area in the HS group compared with the control group, while these changes were dramatically less pronounced in the HS+IR group (Figure 2A). Consistently, aortic mRNA expression of collagen type III and transforming growth factor-β was increased in the HS group but was suppressed in the HS+IR group (Figure 2B,C). There was a marked increase in CD68 staining and gene expression in the aorta of the HS group compared with the control group, but much less compared with the HS+IR group. CD68 positive staining was mainly seen in the adventitia (Figure 2A,D). These results indicate that dietary IR attenuated high salt-induced vascular remodeling.

To clarify the mechanisms by which IR has beneficial effects against the development of hypertension, we evaluated molecular signal pathways in the aorta of the HS+IR group. The phosphorylation of Akt at Ser473 in the aorta was decreased in the HS group compared with the control group, whereas this change was prevented in the HS+IR group (Figure 2E). The phosphorylation of AMPK at Thr172 and eNOS at Ser1177 in the aorta was also decreased in the HS group compared with the HS+IR group; however, these changes were not observed in the HS+IR group (Figure 2F,G). In contrast, the phosphorylation of ERK at Thr202/Tyr204 in the aorta increased in both HS and HS+IR groups compared with the control group (Figure 2H).

**TfR1 Expression Was Increased in the Aorta Under High-Salt Diet**

To investigate how iron intake affects HS-induced hypertension, we evaluated intracellular iron transport proteins, such as TfR1 and ferritin H- and L-subunits in the aorta of these groups. TfR1 is required for the uptake of transferrin-bound iron into the cells. Interestingly, aortic TfR1 gene and protein expression was significantly increased in both HS and HS+IR groups compared with the control group, but the extent to which it increased was higher in the HS group than in the HS+IR group (Figure 3A–C). Immunohistochemical analysis showed that TfR1 was largely expressed in the media (Figure 3C). Aortic ferritin H-subunit gene expression was increased in the HS groups compared with the control group, while ferritin...
The expression of intracellular iron transport proteins in Dahl salt-sensitive rats was investigated. Aortic (A) gene and (B) protein expression of TfR1 in the control (white bar, n = 6), HS (black bar, n = 6), and HS + IR (gray bar, n = 6) groups. C, Representative images of TfR1 staining of the aorta sections. Scale bars: 50 μm. Aortic gene expression of (D) ferritin H- and (E) ferritin L-subunits. (F) protein expression of ferritin H-subunit, and (G) iron contents in the control (white bar, n = 6), HS (black bar, n = 6), and HS + IR (gray bar, n = 6) groups. H, Representative images of DHE staining of the aorta sections. Scale bars: 50 μm. Aortic gene expression of TfR1, ferritin H- and ferritin L-subunits was normalized with glyceraldehyde-3-phosphatedehydrogenasegene expression, and relative levels of gene expression are shown in the graph. Protein expression of TfR1 and ferritin H-subunit was standardized on the basis of β-actin expression, and relative levels of expression are plotted in the graphs. *P < 0.05 vs the control group; †P < 0.05 vs HS group.

**Table 1. Physiological and Echocardiographic Parameters in all Groups at 18 Weeks of Age**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HS</th>
<th>HS + IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV weight/tibia length (mg/mm)</td>
<td>22.2 ± 0.5</td>
<td>32.9 ± 0.9*</td>
<td>28.8 ± 0.7†</td>
</tr>
<tr>
<td>Lung weight/tibia length (mg/mm)</td>
<td>40.5 ± 1.1</td>
<td>108.1 ± 5.3*</td>
<td>42.1 ± 0.7†</td>
</tr>
<tr>
<td>Diastolic wall thickness of LV</td>
<td>1.8 ± 0.0</td>
<td>2.4 ± 0.0*</td>
<td>2.2 ± 0.1†</td>
</tr>
<tr>
<td>posterior wall (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV end-diastolic dimension (mm)</td>
<td>8.2 ± 0.1</td>
<td>8.9 ± 0.1</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>LV end-systolic dimension (mm)</td>
<td>5.2 ± 0.1</td>
<td>5.7 ± 0.4</td>
<td>5.1 ± 0.2</td>
</tr>
<tr>
<td>LV fractional shortening (%)</td>
<td>34.8 ± 1.1</td>
<td>34.6 ± 2.9</td>
<td>39.8 ± 2.5</td>
</tr>
<tr>
<td>Early diastolic filling wave (cm/s)</td>
<td>84.9 ± 5.0</td>
<td>117.2 ± 10.1†</td>
<td>96.8 ± 5.2</td>
</tr>
<tr>
<td>Peak filling velocity at atrial contraction (cm/s)</td>
<td>36.3 ± 2.2</td>
<td>49.0 ± 8.8</td>
<td>72.6 ± 3.0†</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>2.4 ± 0.2</td>
<td>2.8 ± 0.7</td>
<td>1.3 ± 0.1†</td>
</tr>
<tr>
<td>Deceleration time (msec)</td>
<td>39 ± 2</td>
<td>31 ± 1*</td>
<td>46 ± 4†</td>
</tr>
</tbody>
</table>

E/A ratio indicates the ratio of peak early diastolic filling velocity and peak filling velocity at atrial contraction; Control, Dahl salt-sensitive rats fed normal salt diet; HS, Dahl salt-sensitive rats fed high-salt diet; HS + IR, Dahl salt-sensitive rats fed high-salt with iron-restricted diet; LV, left ventricle. *P < 0.05 vs Control group. †P < 0.05 vs HS group. n = 6 per group.

L-subunit gene expression was not increased in the HS group. In contrast, both ferritin H- and L-subunits gene expression was decreased in the HS + IR group compared with the other groups (Figure 3D,E). Aortic ferritin H-subunit protein expression was consistent with the gene expression (Figure 3F). Tissue iron content of the aorta increased in the HS group, while it decreased in the HS + IR group, relative to the control group (Figure 3G).

To investigate further whether dietary IR exerts local antioxidant effect in the aorta, we examined superoxide production by staining of the aorta with DHE. The aorta in the HS group showed a higher fluorescent signal compared with other groups. The HS + IR group showed significantly decreased vascular production of superoxide (Figure 3H).

**Effects of Iron Restriction on Cardiac Function**

As shown in Table 1, HS diet induced a marked increase in left-ventricle (LV)-weight-to-tibia-length ratio compared with the control group at 18 weeks of age, demonstrating cardiac hypertrophy. Lung-weight-to-tibia-length ratio was also increased in the HS group relative to the control group, indicating pulmonary congestion. However, IR + HS diet inhibited the increase in LV-weight-to-tibia-length ratio and lung-weight-to-tibia-length ratio, suggesting that IR attenuated development of heart failure.

Echocardiographic analysis showed that LV hypertrophy was evident at 18 weeks of age in the HS group, whereas it was attenuated in the HS + IR group. LV cavity size and fractional shortening were comparable among the 3 groups. E wave was higher and deceleration time shortened in the HS group compared with the other groups, whereas A wave was higher and deceleration time was prolonged; this resulted in decreasing E/A ratio in the HS + IR group relative to the other groups at 18 weeks of age.

Histological analysis revealed that the cross-sectional area of cardiomyocytes increased in both HS and HS + IR.
groups at 18 weeks of age compared with the control group, but the increase in cross-sectional area of cardiomyocytes was attenuated more in the HS+IR group than in the HS group (Figure 4A,B). In addition, cardiac interstitial fibrosis increased in the HS and HS+IR groups at 18 weeks of age compared with the control group; however, it was reduced significantly in the HS+IR group compared with the HS group (Figure 4A,C); this suggests that IR+HS diet inhibited increased LV hypertrophy and interstitial fibrosis. Consistent with these changes, dietary IR suppressed the increased expression of atrial natriuretic peptide, collagen type I, and CD68 mRNA in the heart of the HS group (Figure 4D–F). These results also supported the finding that dietary IR attenuated the development of LV hypertrophy and heart failure.

**Iron Restriction Attenuated Renal Injury Under High-Salt Diet**

Next, we evaluated proteinuria and urinary 8-Hydroxy-2'-deoxyguanosine (8-OHdG) concentration in these animals. Proteinuria and urinary 8-OHdG/creatinin ratio increased in the HS group compared with the other groups, while they were suppressed in the HS+IR group compared with the HS group (Figure 5A,B). DHE staining showed that there was increased superoxide production in the kidney of the HS+IR group, but the increase in cross-sectional area of cardio-myocyte was reduced significantly in the HS+IR group compared with the other groups; whereas in the HS group, there was decreased renal production of superoxide (Figure 5C). These data indicated that dietary IR inhibited the development of renal injury and oxidative stress under HS diet.

**1-NAME Abolished the Beneficial Effects of Iron Restriction Under High-Salt Diet**

To determine whether IR has beneficial effects through NO-mediated pathway, we next explored the effects of 1-NAME in the HS+IR group. After administration of 1-NAME, SBP markedly increased in the HS+IR group and became similar to that of the HS group (Figure 6A). In addition, 1-NAME treatment abrogated the decreased proteinuria in the HS+IR group (Figure 6B). Consistent with these changes, 1-NAME led to the decreased survival rate in the HS+IR group (Figure 6C). Taken together, 1-NAME abolished the beneficial effects of IR under HS diet. Dietary IR had protective effects on salt-induced organ damage through NO-mediated pathway.

**Discussion**

This study demonstrated that dietary IR has beneficial effects on cardiovascular remodeling in Dahl salt-sensitive rats. IR attenuated the development of hypertension, LV hypertrophy, heart failure, and proteinuria, thereby improving survival rate in Dahl salt-sensitive rats through the inhibition of oxidative stress; it also maintained Akt, AMPK, and eNOS signaling in the aorta. 1-NAME abolished the beneficial effects of IR.
Iron is a vital element in life. However, it may participate in diverse pathological processes by catalyzing the formation of reactive oxygen free radicals. Iron accumulation promotes increased free radicals and oxidative stress, which eventually lead to cell and tissue damage. Therefore, it is important to consider the influence of iron on the pathophysiology of various diseases. Iron is involved in the pathogenesis of several cardiovascular diseases. It has been reported that the iron deposition shown in human atherosclerotic lesions and body iron stores is related to the risk of carotid atherosclerosis. Thus, iron accumulation in atheroma may be associated with the progression of atherosclerosis, and the reduction of iron accumulation may be effective for the development of atherosclerosis. In the current study, we elucidated the effects of dietary IR on cardiovascular remodeling in Dahl salt-sensitive rats. HS-loading on salt-sensitive individuals caused hypertension, LV hypertrophy, heart failure, and renal injury. Of note, we demonstrated that IR attenuated the development of these diseases and improved survival rate in Dahl salt-sensitive rats.

Calorie restriction was previously reported to be beneficial for cardiac remodeling in Dahl salt-sensitive rats; however, there was no report to investigate the effects of only IR against HS-induced cardiac remodeling. To our knowledge, this is the first article to report that only IR was effective for cardiovascular remodeling in Dahl salt-sensitive rats. IR led to only a slight decrease in body weight, consistent with a previous observation, whereas body weight decreased more in the HS group than in the HS+IR group. At 18 weeks of age, Dahl salt-sensitive rats showed cachexia with the development of heart failure, similar to previous reports. Taking these findings into consideration, IR+HS diet prevented the onset of cachexia with the development of heart failure. Although IR improved survival rate in Dahl salt-sensitive heart failure rats, dietary IR induced iron deficiency anemia measured by hemoglobin content, in agreement with previous observation. Interestingly, blood hemoglobin began to decrease in the HS group at 10 weeks of age. Finally, blood hemoglobin was lower in the HS+IR group than in the HS group at 18 weeks of age. Because previous studies have reported that plasma volume increased in Dahl salt-sensitive heart failure rats, several factors such as fluid retention and increased plasma volume seem to influence anemia in the HS group.

Iron deficiency is a contributing factor in heart failure; however, IR attenuated development of heart failure in Dahl salt-sensitive rats. SBP increased in the HS group, while IR inhibited the increase in SBP, despite an HS diet. As a result, it appears that IR attenuated the development of LV hypertrophy, decompensated pressure-overload hypertrophy, and heart failure. To clarify the mechanisms by which IR benefits the development of hypertension, we elucidated molecular signaling pathways in the aorta of these animals. In the current study, the phosphorylation of Akt, AMPK, and eNOS in the aorta decreased in the HS group, whereas it maintained the phosphorylation of Akt, AMPK, and eNOS in the aorta of the HS+IR group. On the contrary, the phosphorylation of ERK in the aorta was increased in both HS and HS+IR groups compared with the control group. Thus, IR appears to prevent the development of hypertension through Akt, AMPK, and eNOS signaling but not through ERK signaling. The phosphorylation of eNOS at Ser 1177 is associated with increased production of NO. Both Akt and AMPK are reported to phosphorylate directly eNOS at Ser 1177, NO production, and vasorelaxation. In addition, cross-talk between Akt and AMPK is reported to be important for eNOS phosphorylation at Ser 1177. Therefore, Akt and AMPK may be upstream kinases of vascular eNOS phosphorylation in the IR-mediated protection of endothelial function. In contrast, oxidative stress also causes endothelial dysfunction through regulation of eNOS. Thus, we investigated the urinary 8-OHdG/creatinin ratio and superoxide production of the aorta with DHE staining among the groups. As expected, the urinary 8-OHdG/creatinin ratio and vascular superoxide production increased in the HS group, whereas IR significantly reduced both systemic and vascular oxidative stress under HS diet. Taken together, these data provide evidence that the attenuation of oxidative stress also contributes to the vascular protective effect of IR. Oxidative stress could regulate the phosphorylation of Akt, AMPK, and eNOS in the aorta, whereas aortic ERK signaling is reported not to be dependent on the attenuation of oxidative stress in the...
hypertensive rats. Therefore, we may observe differential response between the signaling axis of Akt, AMPK, eNOS, and that of ERK.

To investigate how iron intake affects HS-induced hypertension, we evaluated cellular iron transport proteins, such as TIR1 and ferritin H- and L-subunits, in the aorta of these animals. Low-iron conditions normally lead to upregulated TIR1 and downregulated ferritin expression. Conversely, high-iron conditions usually downregulate TIR1 and upregulate ferritin expression. We observed these changes in the aorta of the HS + IR group as normal, while this was not the case in the HS group. Interestingly, both TIR1 and ferritin H-subunit expression were upregulated in the aorta of the HS group, suggesting that dysregulation of cellular iron transport proteins occurs in the aorta of the HS group. More iron is necessary for cells for their growth and metabolism than for resting cells. Upregulated aortic TIR1 may increase iron uptake into the cell and participate in vascular remodeling in the HS group. Since oxidative stress was reported to upregulate TIR1 expression, oxidative stress may be related to aortic TIR1 upregulation in the HS group. Meanwhile, ferritin is a major intracellular iron-storage protein. Ferritin H-subunit has ferroxidase activity, which is required for iron sequestration, while ferritin L-subunit has no ferroxidase activity. Only ferritin H-, but not L-subunit, expression was increased in the aorta of the HS group, which may sequestrate excess free iron molecules to minimize generation of iron-catalyzed reactive oxygen species.

Clinically, treatment with intravenous iron improved exercise capacity and symptoms of heart failure in patients with or without anemia, although excess total body iron stores are associated with higher cancer risk and mortality. In this study, both HS and HS + IR group showed anemia, whereas the HS + IR group showed good prognosis. Iron is essential for several biological processes, such as enzymatic reactions and oxygen delivery, while its excess is involved in pathophysiology. Therefore, although iron supplementation may be beneficial for patients with heart failure, the role of iron in maintaining cardiac function in hypoxia and anemia has not been further considered. In addition, studies using intravenous iron treatment are needed to assess long-term safety in renal and cardiac disease.

In conclusion, dietary IR has protective effects on HS-induced hypertension and cardiovascular remodeling through the reduction of oxidative stress and maintenance of Akt, AMPK, and eNOS signaling in the aorta. IR could be an effective strategy for prevention of HS-induced organ damage in salt-sensitive hypertensive patients.

Perspectives
We have shown that dietary IR has preventive effects on salt-induced cardiovascular remodeling in Dahl salt-sensitive rats. In addition, we found that dysregulation of intracellular iron transport proteins, such as upregulation of TIR1 and ferritin H-subunit occurs in the aorta of Dahl salt-sensitive rats with an HS diet. This is the first article to our knowledge that reveals the effect of dietary IR on hypertensive cardiovascular remodeling. Notably, we have shown that IR attenuated the development of hypertension, LV hypertrophy, heart failure, and renal injury, thereby improving survival rate in Dahl salt-sensitive rats; this occurred through inhibition of oxidative stress and by maintaining Akt, AMPK, and eNOS signaling in the aorta. Based on our findings, understanding the beneficial effects of dietary IR on salt-induced cardiovascular remodeling may lead to a new therapeutic strategy for prevention of HS-induced organ damage in salt-sensitive hypertensive patients.

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Disclosures
None.

References


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Dietary Iron Restriction Prevents Hypertensive Cardiovascular Remodeling in Dahl Salt-Sensitive Rats

Short title: Iron restriction and salt-sensitive hypertension

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Methods

Assessments of Blood Pressure, Blood Cell Count, Urinary 8-Hydroxy-2'-deoxyguanosine, and Tissue Iron Content: Systolic blood pressure and heart rate were measured by a non-invasive computerized tail-cuff system in conscious rats (MK-2000, Muromachi Kikai, Tokyo, Japan) (1). Peripheral blood cell count was measured using an automatic cell count analyzer (Pentra 60 LC-5000, Horiba, Kyoto, Japan). 24-hour urine samples were collected in metabolic cages for measuring urinary volume, protein, and 8-Hydroxy-2'-deoxyguanosine (8-OHdG) and creatinine levels. Urinary 8-OHdG levels were assessed by enzyme-linked immune sorbent assay (Japan Institute for the Control of Aging, Shizuoka, Japan). The iron content of the aorta was determined by atomic absorption.

Echocardiography: Rats were anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and assessed by transthoracic echocardiography (Aplio, Toshiba Medical Systems Co., Odawara, Japan). Left ventricle (LV) cavity size, wall thickness, and LV fractional shortening were calculated. We also measured peak early diastolic filling velocity (E), peak filling velocity at atrial contraction (A), their ratio (E/A) and deceleration time from pulsed Doppler mitral flow velocity pattern (2).

Gene Expression Analysis: Total RNA was extracted from the tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) (3). DNase-treated RNA was reverse-transcribed into cDNA using random primers (Applied Biosystems, Foster City, CA, USA). Real-time PCR reactions were performed using the ABI PRISM 7900 with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) (1). TaqMan Gene Expression Assays were used as primers and probes for each gene were as follows: atrial natriuretic peptide (assay ID Rn00561661_m1), collagen type I (assay ID Rn00801649_g1), collagen type III (assay ID Rn01437683_m1), transforming growth factor-β (assay ID Rn99999016_m1), CD68 (assay ID Rn01495634_g1), transferrin receptor1 (assay ID Rn01474701_m1), ferritin H-subunit (assay ID Rn00820640_g1), ferritin L-subunit (assay ID Rn00821071_g1), and Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (assay ID Rn99999916_s1). GAPDH was used as an internal control.

Histological Analysis: Heart tissues were fixed with buffered 4% paraformaldehyde, embedded in paraffin, and cut into 4-µm-thick sections. Hematoxylin-eosin and Masson’s trichrome staining were performed using serial sections. Photomicrographs
were quantified with the use of NIH Image-J software to measure the cross-sectional area of cardiomyocytes and to assess the fibrosis area of myocardium. 100 randomly selected cardiomyocytes in the LV were measured for cross-sectional area (1).
References

