Exercise Training Alters Left Ventricular Geometry and Attenuates Heart Failure in Dahl Salt-Sensitive Hypertensive Rats

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Abstract—The clinical efficacy of exercise training in individuals with heart failure is well established, but the mechanism underlying such efficacy has remained unclear. An imbalance between cardiac hypertrophy and angiogenesis is implicated in the transition to heart failure. We investigated the effects of exercise training on cardiac pathophysiology in hypertensive rats. Dahl salt-sensitive rats fed a high-salt diet from 6 weeks of age were assigned to sedentary or exercise (swimming)-trained groups at 9 weeks. Exercise training attenuated the development of heart failure and increased survival, without affecting blood pressure, at 18 weeks. It also attenuated left ventricular concentricity without a reduction in left ventricular mass or impairment of cardiac function. Interstitial fibrosis was increased and myocardial capillary density was decreased in the heart of sedentary rats, and these effects were attenuated by exercise. Exercise potentiated increases in the phosphorylation of Akt and mammalian target of rapamycin observed in the heart of sedentary rats, whereas it inhibited the downregulation of proangiogenic gene expression apparent in these animals. The abundance of the p110α isoform of phosphatidylinositol 3-kinase 3-kinase was decreased, whereas those of the p110γ isoform of phosphatidylinositol 3-kinase and the phosphorylation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase were increased, in the heart of sedentary rats, and all of these effects were prevented by exercise. Thus, exercise training had a beneficial effect on cardiac remodeling and attenuated heart failure in hypertensive rats, with these effects likely being attributable to the attenuation of left ventricular concentricity and restoration of coronary angiogenesis through activation of phosphatidylinositol 3-kinase(p110α)-Akt-mammalian target of rapamycin signaling. (Hypertension. 2009;53:701-707.)

Key Words: hypertension □ sodium-dependent □ heart failure □ exercise □ hypertrophy □ rats □ Dahl □ coronary angiogenesis

Heart failure is a final common consequence of various forms of heart disease and is a leading cause of mortality worldwide. Cardiac hypertrophy associated with pathological conditions such as hypertension, myocardial infarction, and valvular heart disease has been thought to be an adaptive response to increased external load, given that it can result in normalization of the increase in wall stress induced by mechanical overload. However, increased cardiac mass is also associated with increased morbidity and mortality, with sustained overload eventually leading to heart failure.

The serine-threonine protein kinase Akt is an important mediator of phosphatidylinositol 3-kinase (PI3K) signaling and regulates multiple cellular functions. Akt signaling is implicated in the regulation of cardiac growth, contractile function, and coronary angiogenesis. A mismatch between the number of coronary capillaries and the size of cardiomyocytes, resulting in myocardial hypoxia, is thought to develop during the progression of cardiac hypertrophy. Indeed, studies have indicated the existence of a relation among cardiac angiogenesis, hypertrophy, and function. Attenuation of coronary angiogenesis in the setting of load-induced cardiac growth may, thus, play an important role in the development of cardiac pathophysiology, with the balance between cardiac growth and coronary angiogenesis, rather than the extent of hypertrophy, being a key determinant of the transition from physiological to pathological hypertrophy. Antiangiogenic activity of the tumor suppressor protein p53 has been implicated recently in the transition from cardiac hypertrophy to heart failure.
programs of exercise in patients with heart failure are generally safe and may improve exercise tolerance, vascular endothelial function, central cardiac function, and overall quality of life. Exercise training also appears to improve survival in patients or animal models with heart failure. However, the mechanism underlying such efficacy has remained unclear.

We have now investigated the effects of exercise training on cardiac growth, contractile function, and coronary angiogenesis, as well as on PI3K-Akt signaling in a rat model of hypertension-induced heart failure. We hypothesized that exercise training might alter left ventricular (LV) geometry and induce myocardial angiogenesis and that such effects might contribute to amelioration of heart failure.

Methods

Animals and Experimental Protocols
Male inbred Dahl salt-sensitive (DS) rats fed an 8% NaCl diet after 6 weeks of age were assigned to sedentary (HF; n=16) or exercise-trained (Ex; n=8) groups at 9 weeks. Exercise training consisted of swimming for 1 hour per day, 5 days per week, for 9 weeks. DS rats maintained on a 0.3% NaCl diet after 6 weeks of age remain normotensive, and such animals served as age-matched controls (CNT group; n=8). At 18 weeks of age, all of the rats were anesthetized by injection of ketamine (50 mg/kg of body weight) and xylazine (10 mg/kg) and were subjected to echocardiographic and hemodynamic analyses. The heart was subsequently excised, and LV tissue was separated for analysis.

Tissue Preparation
For details, please see the online data supplement (available at http://hyper.ahajournals.org).

Echocardiographic and Hemodynamic Analyses
Systolic blood pressure (SBP) and heart rate (HR) were measured weekly in conscious animals by tail-cuff plethysmography (BP-98A; Softron). At 18 weeks of age, rats were subjected to transthoracic echocardiography, as described previously. Details of echocardiographic analysis are available in the online data supplement. After echocardiography, cardiac catheterization was performed as described previously. Tracings of LV pressure and the ECG were digitized to determine LV end-diastolic pressure.

Histology and Immunohistochemistry
The left ventricle was fixed in ice-cold 4% paraformaldehyde for 48 to 72 hours, embedded in paraffin, and processed for histology and immunohistochemistry, as described. Sections were stained with mouse monoclonal antibodies to the endothelial cell marker CD31 (diluted 1:100; Pharmingen) to determine the extent of coronary capillary formation. Individual endothelial cells or clusters of endothelial cells, with or without a lumen, were regarded as capillaries. Capillary density was expressed as the average number of capillaries per square millimeter. The ratio of the number of coronary capillaries to that of cardiomyocytes was also determined. All of the image analysis was performed with National Institutes of Health Scion Image software. Details are available in the online data supplement.

Quantitative RT-PCR Analysis
Total RNA was extracted from LV tissue and subjected to quantitative RT-PCR analysis, as described, with primers and TaqMan probes specific for rat complementary DNA encoding hypoxia-inducible factor (HIF) 1α (5′-ACTGCACGAGCCACATTCTAG-3′, 5′-CCAGCACAAGCAGCTCATAGG-3′, and 5′-ACCAGCAGTAA-CCACCCGACTG-3′ as the forward primer, reverse primer, and TaqMan probe, respectively; GenBank accession No. NM.024359), vascular endothelial growth factor (VEGF), and endothelial NO synthase. Reagents for detection of human 18S rRNA (Applied Biosystems) were used to quantify rat 18S rRNA as an internal standard. Details are available in the online data supplement.

Immunoblot Analysis
Total protein was isolated from LV tissue and quantitated with the Bradford reagent (Bio-Rad). Equal amounts of the total protein fraction were subjected to SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane, as described previously. The membrane was incubated with a 1:1000 dilution of rabbit polyclonal antibodies to the p110α isoform of PI3K, the p110y isoform of PI3K, Akt, Akt phosphorylated on Ser473, mammalian target of rapamycin (mTOR), mTOR phosphorylated on Ser2448, p70 S6 kinase, p70 S6 kinase phosphorylated on Thr389, p38 mitogen-activated protein kinase (MAPK), p38 MAPK phosphorylated on Thr180 and Tyr182, extracellular signal-regulated kinase (ERK) 1 and 2, or ERK1/2 phosphorylated on Thr202 and Tyr204 (Cell Signaling Technology) and a dilution of a goat polyclonal antibody to GAPDH (Santa Cruz Biotechnology). It was then exposed to a 1:1000 dilution of horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G (Medical and Biological Laboratories), after which immune complexes were detected and quantified as described previously.

Statistical Analysis
Data are presented as means±SEMs. Differences among groups were assessed by 1-way factorial ANOVA; if a significant difference was detected, intergroup comparisons were performed with Fisher’s multiple-comparison test. The time courses of SBP and HR were compared among groups by 2-way, repeated-measures ANOVA. Survival rate was analyzed by the standard Kaplan–Meier method with a log-rank test. A P value of <0.05 was considered statistically significant.

Results

LV Geometry, Cardiac Function, and Survival
SBP was significantly higher in the HF group than in the CNT group at 7 weeks of age and thereafter (Figure 1A and Table 1). It was slightly lower in the Ex group than in the HF group from 9 weeks of age, when the animals in the Ex group began exercise training, through 12 weeks of age. However, there were no significant differences in SBP between the HF and Ex groups from 6 through 18 weeks. HR was significantly higher in the HF group than in the CNT group at 7 weeks of age and thereafter (Figure 1B and Table 1). The Ex group showed a significant reduction in HR from 10 to 12 weeks compared with the HF group. However, HR in the Ex group began to increase again at 13 weeks and continued to do so until 18 weeks, when it was almost the same as that in the HF group. At 18 weeks of age, the ratio of LV weight:tibial length, an index of cardiac hypertrophy, was increased by 58% in the HF group compared with the CNT group (Table 1). The ratio of lung weight:tibial length, an index of pulmonary congestion, was also increased by 24% in the HF group (Table 1). Exercise training did not affect the increase in the LV weight:tibial length ratio, but it prevented that in the lung weight:tibial length ratio. Although 9 (56%) of 16 rats died in the HF group during the experimental period (6 from heart failure and 3 before the development of heart failure, probably from lethal arrhythmia), only 1 (13%) of 8 rats did so (from heart failure) in the Ex group (Table 1). Kaplan–Meier analysis confirmed that the survival rate of exercised rats was significantly greater than that of HF rats (Figure 1C).
Body weight, g 410 ± 11 367 ± 4* 367 ± 3*
SBP, mm Hg 130 ± 5 213 ± 3* 217 ± 5*
HR, bpm 379 ± 5 413 ± 4* 408 ± 1†
Heart weight/TI, mg/mm 28.6 ± 0.6 41.6 ± 0.7* 41.6 ± 1.1*
LV weight/TI, mg/mm 20.0 ± 0.5 31.5 ± 0.7* 31.3 ± 0.8*
Lung weight/TI, mg/mm 36.1 ± 0.8 44.7 ± 1.9* 39.7 ± 0.5†
LVEDP, mm Hg 4.1 ± 0.2 8.3 ± 1.2* 4.8 ± 0.6†
Survival, % 100 44* 88†

**TL** indicates tibial length; **LVEDP**, LV end-diastolic pressure. Data are means ± SEMs unless otherwise specified.

*P<0.05 vs CNT group.
†P<0.05 vs HF group.

Exercise training did not affect LV mass, but it significantly attenuated the changes in interventricular septum thickness, LV posterior wall thickness, LV fractional shortening, RWT, and LV end-diastolic dimension. Hemodynamic analysis revealed that LV end-diastolic pressure was significantly increased in the HF group compared with the CNT group and that exercise training attenuated the load-induced increase in LVEDP (Table 1). Hemodynamic overload resulted in a rightward and upward shift in the plot of RWT versus LV mass, whereas exercise training resulted in a downward shift with a slight shift to the left in this plot (Figure 2B). These data indicate that exercise training attenuated LV concentricity without affecting LV mass or impairing cardiac function.
were prevented by exercise training (Figure 4A and 4B). The myocardial abundance of the p110α isoform of PI3K was significantly increased, in the HF group compared with the CNT group. These changes in PI3K isoform expression was significantly reduced by exercise training (Table 2). CD31 immunostaining for coronary capillaries. Scale bars, 50 μm. B and C, Capillary density (B) and the ratio of the number of capillaries to that of myocytes (C) in cross sections of the LV myocardium. Data are means±SEMs (n=8, 7, and 7 for the CNT, HF, and Ex groups, respectively). *P<0.05 vs CNT group; †P<0.05 vs HF group.

Cardiac Fibrosis and Coronary Angiogenesis
Histological analysis revealed marked interstitial fibrosis in the left ventricle of rats in the HF group compared with those in the CNT group. This increase in cardiac fibrosis was significantly reduced by exercise training (Figure 3).

Immunostaining of the myocardium with antibodies to CD31 to detect capillary endothelial cells revealed that capillary density was decreased in the HF group as a result of the pronounced cardiac hypertrophy and that exercise training restored capillary density to the level apparent in the CNT group despite the remaining cardiac hypertrophy (Figure 3A and 3B). The ratio of the number of coronary capillaries to that of cardiomyocytes was slightly but significantly increased in the HF group compared with the CNT group, and exercise training induced an additional increase in this ratio (Figure 3C).

Activation of Proangiogenic Signaling
The myocardial abundance of the p110α isoform of PI3K was markedly reduced, whereas that of the p110γ isoform of PI3K was significantly increased, in the HF group compared with the CNT group. These changes in PI3K isoform expression were prevented by exercise training (Figure 4A and 4B). The ratio of the amount of the phospho-Ser473 form of Akt to that of total Akt was significantly increased in the HF group compared with the CNT group, and exercise training resulted in a further increase in this ratio (Figure 4C). The phosphorylation of mTOR on Ser2448 was also increased in the HF group compared with the CNT group and was increased further by exercise training (Figure 4D). The phosphorylation of p70 S6 kinase on Thr389 was significantly increased in the HF group compared with the CNT group, but it was not affected further by exercise training (Figure 4E). The phosphorylation levels of both p38 MAPK and ERK1/2 were significantly increased in the HF group compared with the CNT group, and these effects were prevented by exercise training (Figure 4F and 4G). Finally, the amounts of HIF-1α, VEGF, and endothelial NO synthase mRNAs in the left ventricle were markedly reduced in the HF group compared with the CNT group, and these changes were prevented by exercise training (Figure 5).

Discussion
We have shown that exercise training altered LV geometry, restored coronary capillary growth, and attenuated the development of decompensated heart failure in hypertensive DS rats. The beneficial effects of exercise training in this model are likely attributable, at least in part, to amelioration of the imbalance between cardiac growth and angiogenesis through activation of the PI3K(p110α)-Akt-mTOR signaling pathway.

Exercise training did not significantly affect SBP during the experimental period, although the increase in SBP induced by the high-salt diet was slightly attenuated for several weeks after the initiation of exercise, consistent with previous observations in spontaneously hypertensive rats and heart failure rats. Exercise training increases venous return, leading to an increase in cardiac output, contributing to the preserved hypertension, especially in the setting of high salt intake. The responses of SBP to exercise, thus, likely depend on the form and the intensity and duration of exercise or on the extent of chronic volume load. HR was significantly lowered by exercise training during the initial few weeks. Exercise training increases baroreflex and overall vagal activity, and a slow HR at rest is in general associated with greater longevity. Indeed, exercise training inhibited the development of heart failure and increased survival rate in the present model of hypertension, consistent with the results of previous studies showing that exercise training induces bradycardia and improves survival in hypertensive rat models. The return to an increasing HR at 13 weeks in the Ex group in the present study might be attributable to the progression of cardiac pathophysiology resulting from persistent severe hypertension.

The adaptations induced by exercise training, including physiological hypertrophy, may be regarded as compensatory responses to a chronic volume load. Patients with essential hypertension manifest 4 different patterns of LV geometry in terms of LV mass and RWT. Mortality and the frequency of cardiovascular events are highest in patients with concentric hypertrophy and intermediate in those with eccentric hypertrophy. In the present study, exercise training attenuated LV concentricity without affecting LV mass or impairing cardiac function. It thereby attenuated heart failure and increased survival in hypertensive DS rats, consistent with previous observations with other animal models of hypertension. The physiological form of cardiac hypertrophy is an adaptive response to long-term exercise training, whereas the pathological form is often a maladaptive response to provocative stimuli, such as hypertension and aortic stenosis.
physiological hypertrophy apparent in the heart of athletes manifests as eccentric hypertrophy. The progression to heart failure is typically associated with increased fibrosis and disruption of normal cellular organization in the LV myocardium. Exercise training reduced the extent of interstitial fibrosis in the left ventricle of hypertensive DS rats, consistent with the notion that physiological hypertrophy is not associated with interstitial fibrosis. The attenuation of LV concentricity observed in the Ex group, thus, likely reflects a compensatory morphological response to maintain cardiac function.

The balance between cardiac growth and coronary angiogenesis is a key determinant of cardiac function, with disturbance of this balance being implicated in the transition from adaptive hypertrophy to heart failure. Physiological cardiac hypertrophy is associated with a normal or increased number of myocardial capillaries, whereas pathological hypertrophy is associated with a reduction in capillary density. Indeed, myocardial capillary density is reduced in patients with heart disorders, eg, aortic stenosis, dilated cardiomyopathy, or ischemic cardiomyopathy. In the present study, exercise training reduced the extent of cardiac interstitial fibrosis and restored coronary capillary density, as well as attenuated LV concentricity in hypertensive DS rats. In addition, exercise training induced a further increase in the ratio of the number of coronary capillaries to that of cardiomyocytes in these rats.

Figure 4. Abundance of p110α and p110γ isoforms of PI3K, as well as phosphorylation of Akt, mTOR, p70 S6 kinase, p38 MAPK, and ERK, in the left ventricle of DS rats in the 3 experimental groups at 18 weeks of age. Representative immunoblots and either the ratio of the amounts of p110α (A) or p110γ (B) to that of GAPDH or the ratio of phosphorylated (p-) to total forms of Akt (C), mTOR (D), p70 S6 kinase (p70S6K; E), p38 MAPK (F), or ERK1/2 (G) are shown. Quantitative data are expressed relative to the corresponding value for the CNT group and are means±SEMs (n=8, 7, and 7 for the CNT, HF, and Ex groups, respectively). *P<0.05 vs CNT group; †P<0.05 vs HF group.

Figure 5. Expression of proangiogenic genes in the left ventricle of DS rats in the 3 experimental groups at 18 weeks of age. The abundance of HIF-1α (A), VEGF (B), and endothelial NO synthase (eNOS; C) mRNAs was determined by RT-PCR analysis, normalized by that of 18S rRNA, and then expressed relative to the normalized value for the CNT group. Data are means±SEMs (n=8, 7, and 7 for the CNT, HF, and Ex groups, respectively). *P<0.05 vs CNT group; †P<0.05 vs HF group.
animals. These data are consistent with those of previous studies showing that capillary density was increased in the hypertrophic left ventricle by exercise training.²¹,³³ The exercise-induced normalization of the imbalance between cardiac growth and coronary angiogenesis may, thus, have contributed to the preservation of cardiac function and the consequent improvement in the survival of hypertensive DS rats.

Akt is activated by various extracellular stimuli in a PI3K-dependent manner and regulates multiple aspects of cellular functions, including survival, growth, and metabolism.² Akt is required for physiological cardiac growth.³ However, in Akt transgenic mice, long-term Akt activation results in excessive cardiac hypertrophy associated with pathological remodeling and loss of contractile function.²⁹ Akt is activated by the p110α isoform of PI3K in the induction of physiological hypertrophy, but it is also activated by the p110γ isoform of PI3K in response to various agonists of G protein–coupled receptors, eg, endothelin 1, in the induction of pathological hypertrophy.⁴ It is, therefore, unlikely that PI3K-Akt signaling is the sole determinant of physiological versus pathological hypertrophy. It is possible, however, that the intensity or duration of signaling mediated by p110α differs from that mediated by p110γ and that such a difference contributes to the induction of physiological versus pathological hypertrophy.³⁴ In the present study, the abundance of p110α was markedly reduced, whereas that of p110γ was significantly increased, in the left ventricle of HF rats. In addition, the increase in the phosphorylation levels of Akt and mTOR in HF rats was not accompanied by an increase in myocardial capillary density. Furthermore, the activation of p38 MAPK and ERK apparent in the HF group is indicative of the development of pathological hypertrophy.⁵,³⁴ In contrast, exercise training inhibited the isoform shift of PI3K, as well as the activation of p38 MAPK and ERK, with these effects likely underlying both the prevention of the decrease in myocardial capillary density and the further increase in the extents of Akt and mTOR phosphorylation. Exercise activates the insulin-like growth factor 1-PI3K(p110α) pathway, and it has been shown that PI3K(p110α) plays a critical role in the induction of cardiac growth induced by exercise training.⁵ The p110α isoform of PI3K has also been suggested to inhibit signaling at the level of G protein–coupled receptors or G proteins.⁴ It is, thus, likely that physiological stimuli (eg, exercise training) cannot only switch on the IGF1-PI3K(p110α) pathway but also switch off the G protein–coupled receptor agonist PI3K(p110γ) pathway activated by pathological stimuli (eg, pressure overload).⁴ Together, these observations suggest that exercise training reduced the imbalance between cardiac growth and angiogenesis through activation of the PI3K(p110α)-Akt-mTOR pathway.

VEGF is a central regulator of angiogenesis. Akt-induced expression of VEGF is mediated by activation of mTOR and consequent upregulation of HIF-1.³⁵ The mTOR-dependent expression of VEGF is upregulated during the physiological phase of cardiac growth but is downregulated during the pathological phase of cardiac hypertrophy.²⁹ In the present study, the increase in the phosphorylation of mTOR was accompanied by phosphorylation of p70 S6 kinase in the left ventricle of HF rats. However, the abundance of HIF-1α, VEGF, and endothelial NO synthase mRNAs was reduced in the left ventricle of these animals. Exercise-induced stimulation of Akt-mTOR signaling was associated with restoration of the expression of the genes for these proangiogenic proteins. The phosphorylation of p70 S6 kinase was not affected by exercise training. These data suggest that short-term Akt activation promotes coronary angiogenesis in a manner dependent on mTOR and that long-term activation of Akt-mTOR signaling results in its uncoupling from stimulation of the expression of proangiogenic proteins, leading to impaired coronary angiogenesis and excessive growth of the heart.²⁹ The underlying mechanisms responsible for exercise-induced restoration of coronary angiogenesis remain to be determined. It is possible that the increase in coronary blood flow and shear stress induced by exercise training improves vascular endothelial function or that the antioxidant or anti-inflammatory effects of exercise training result in inhibition of myocardial fibrosis and stimulation of coronary capillary formation.

With regard to limitations of the present study, we did not assess whether the intensity of exercise used was the most appropriate in this model of hypertension. The duration of exercise training was based on previous observations,²¹ but the efficacy of shorter durations of exercise has also been demonstrated.¹³,¹⁴ Excessive long-term exercise was shown to promote cardiac fibrosis and to have deleterious effects on cardiac remodeling²⁸,³⁶ or to lead to bronchial congestion resulting from increased left atrial pressure,³⁷ possibly accelerating the progression to heart failure. Recently, the Heart Failure and a Controlled Trial Investigating Outcomes of Exercise Training Trial demonstrated that an exercise training program in patients with heart failure is safe and may contribute to the reduction of clinical events but does not improve the short-term survival.³⁸ Exercise regimens must, therefore, be carefully calibrated for clinical application to heart failure.²⁹

**Perspectives**

We have shown that exercise training had a beneficial effect on cardiac remodeling and attenuated heart failure in hypertensive DS rats. It promoted coronary angiogenesis mediated by PI3K(p110α)-Akt-mTOR signaling and attenuated LV concentricity and inhibited myocardial fibrosis, leading to the preservation of cardiac function and improved survival. Given that the balance between cardiac growth and angiogenesis is a key determinant of cardiac function, it may be advantageous to stimulate angiogenesis as part of a general strategy to prevent or reverse heart failure. It may be possible to treat hypertension and heart failure with a combination of antihypertrophic (antihypertensive drugs) and proangiogenic (exercise training) protocols, with the combined therapy being more effective than either treatment alone.

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Disclosures
None.

References
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EXERCISE TRAINING ALTERS LEFT VENTRICULAR GEOMETRY AND ATTENUATES HEART FAILURE IN DAHL SALT-SENSITIVE HYPERTENSIVE RATS

Short title: Exercise and heart failure

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I. Expanded Materials and Methods

Animals and Experimental Protocols
Male inbred Dahl salt-sensitive (DS) rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were handled in accordance with the guidelines of Nagoya University Graduate School of Medicine as well as with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). Weaning rats were fed laboratory chow containing 0.3% NaCl until 6 weeks of age. DS rats fed an 8% NaCl diet after 6 weeks manifest hypertension-induced compensated cardiac hypertrophy at 11 weeks and a distinct stage of decompensated heart failure at 18 weeks. Exercise training consisted of swimming for 1 h per day on 5 days per week for 9 weeks. The rats swam in a pool (50 × 50 × 70 cm) filled with water to a depth of 50 cm. The water temperature was maintained at 36° ± 1°C. Swimming is thought to be a natural behavior of rodents, and in addition, it is less stressful than, and avoids electric shock and foot injury associated with, other forced exercise protocols. Both the diets and tap water were provided ad libitum throughout the experimental period.

Echocardiographic Analysis
M-mode echocardiography was performed with a 12.5-MHz transducer (Aplio SSA-700A; Toshiba, Tochigi, Japan). LV end-diastolic (LVDd) and end-systolic (LVDs) dimensions and the thickness of the interventricular septum (IVST) and LV posterior wall (LVPWT) were measured, and LV fractional shortening (LVFS), relative wall thickness (RWT), and LV mass were calculated as follows: LVFS (%) = [(LVDd – LVDs)/LVDd] × 100; RWT = (IVST + LVPWT)/LVDd; LV mass (g) = ({1.04 × [(IVST + LVDd + LVPWT)3 – (LVDd)3]} × 0.8) + 0.14.1

Tissue Preparation
The left ventricle was cut transversely into four portions comprising the apex, two middle rings (each 3 mm in thickness), and the base. For histology, one of the middle-ring specimens was fixed in ice-cold 4% paraformaldehyde for 48 to 72 h and then embedded in paraffin; for preparation of frozen sections, the other middle-ring specimen was rapidly frozen in isopentane cooled with liquid nitrogen and was then embedded in OCT compound within a cryomold. The apex specimen was rapidly frozen in liquid nitrogen and stored at –80°C until analysis.

Histology and Immunohistochemistry
Sections were cut from paraffin blocks at a thickness of 3 μm and were stained with Azan Mallory solution for evaluation of fibrosis, as described previously.2 To determine the extent of coronary capillary formation, we performed immunostaining for the endothelial cell marker CD31 with frozen sections (thickness, 5 μm) that had been fixed with acetone. Endogenous peroxidase activity was blocked by exposure of the sections to methanol containing 0.3% hydrogen peroxide. Sections were incubated at 4°C first overnight with mouse monoclonal antibodies to CD31 (diluted 1:100; Pharmingen, San Diego, CA) and then for 30 min with Histofine Simple Stain Rat MAX PO (Nichirei Biosciences, Tokyo, Japan). Immune complexes were visualized with diaminobenzidine and hydrogen peroxide, and the sections were counterstained with hematoxylin. Capillaries and surrounding cardiomyocytes were counted in five different microscopic fields (×400) of each section.

Quantitative RT-PCR Analysis
Total RNA was extracted from LV tissue and treated with DNase with the use of an SV Total RNA Isolation System (Promega, Madison, WI). Complementary DNA (cDNA) was synthesized by reverse transcription (RT) from 2 μg of total RNA with the use of random primers (Invitrogen, Carlsbad, CA) and MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reaction (PCR) analysis was performed with a Prism 7700 Sequence Detector (Perkin-Elmer, Waltham, MA), with primers and TaqMan probes specific for rat cDNAs encoding hypoxia-inducible factor (HIF)-1α.
(5'-ACTGCACAGCCACATTCTATG-3', 5'-CAGCACCAAGCAGCAGTCATAGG-3', and 5'-ACCAGCAGTAACCAGCGCAGTGTG-3' as the forward primer, reverse primer, and TaqMan probe, respectively; GenBank accession no. NM_024359), vascular endothelial growth factor (VEGF),\(^3\) and endothelial nitric oxide synthase (eNOS).\(^4\) Reagents for detection of human 18S rRNA (Applied Biosystems) were used to quantify rat 18S rRNA as an internal standard.

References