Role of Xanthine Oxidoreductase in the Reversal of Diastolic Heart Failure by Candesartan in the Salt-Sensitive Hypertensive Rat

Eiichiro Yamamoto, Keiichiro Kataoka, Takuro Yamashita, Yoshiko Tokutomi, Yi-Fei Dong, Shinji Matsuba, Hisao Ogawa, Shokei Kim-Mitsuyama

Abstract—The role of angiotensin II and reactive oxygen species in the exacerbation of diastolic heart failure is unknown. We examined the therapeutic effect of angiotensin blockade on hypertensive diastolic heart failure, focusing on the role of xanthine oxidoreductase and reduced nicotinamide-adenine dinucleotide phosphate oxidase, major enzymes producing reactive oxygen species. Dahl salt-sensitive hypertensive rats (DS rats) with established diastolic heart failure were given vehicle, candesartan (an angiotensin II receptor subtype 1 receptor blocker), oxypurinol (a xanthine oxidoreductase inhibitor), apocynin (a reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor), or hydralazine (a vasodilator), and their therapeutic effects on diastolic heart failure were compared. Candesartan treatment of DS rats with established diastolic heart failure reversed cardiac remodeling, improved cardiac relaxation abnormality, and prolonged survival, being accompanied by the attenuation of the increase in cardiac superoxide, reduced nicotinamide-adenine dinucleotide phosphate oxidase, and xanthine oxidoreductase activities. Thus, the beneficial effect of candesartan in DS rats appears to be mediated by the inhibition of cardiac reactive oxygen species. Cardiac xanthine oxidoreductase inhibition with oxypurinol significantly reduced cardiac superoxide, prevented the progression of cardiac remodeling, and delayed the mortality in DS rats. Apocynin, which significantly inhibited cardiac reduced nicotinamide-adenine dinucleotide phosphate oxidase activity, prevented the exacerbation of diastolic heart failure more than hydralazine. However, compared with candesartan or oxypurinol, apocynin did not improve cardiac reactive oxygen species, remodeling, and function in DS rats. In conclusion, candesartan slowed the exacerbation of hypertensive diastolic heart failure in DS rats by causing reverse cardiac remodeling. Candesartan xanthine oxidoreductase contributed to these beneficial effects of candesartan. (Hypertension. 2007;50:657-662.)

Key Words: angiotensin II hypertension diastolic heart failure xanthine oxidoreductase NADPH oxidase

Although the detailed mechanism of systolic heart failure has been extensively investigated, the mechanism and the therapeutic strategy of diastolic heart failure are poorly understood. Accumulating clinical and experimental evidence indicate that blockers of the renin-angiotensin system, such as angiotensin II receptor subtype 1 receptor blockers (ARB) and angiotensin-converting enzyme inhibitors, are the useful therapeutic agents for systolic heart failure. However, the clinical use of renin-angiotensin system blockers for diastolic heart failure remains to be defined. Interestingly, the Candesartan in Heart Failure: Assessment of Reduction in Mortality and Morbidity-Preserved Trial reveals that candesartan prevents admissions for congestive heart failure among patients who have heart failure and left ventricular (LV) ejection fraction >40%, findings suggesting that ARB may be effective for treatment of diastolic heart failure. Dahl salt-sensitive hypertensive rats (DS rats) are the useful model of not only salt-sensitive hypertension but also hypertensive diastolic heart failure. Interestingly, DS rats, fed high salt from 7 weeks of age progressively develop hypertension and exhibit overt diastolic heart failure at 20 weeks of age. Therefore, DS rats are regarded as the useful model to investigate the use of pharmacological intervention for hypertensive diastolic heart failure. We and other groups of investigators have reported previously that ARB markedly prevents the onset of diastolic heart failure in DS rats when the medication is administered at an earlier stage than diastolic heart failure. However, the information on the effect of ARB on established diastolic heart failure is scarce and the precise mechanism responsible for the exacerbation of hypertensive diastolic heart failure remains to be elucidated.

A growing body of evidence reveals the implication of reactive oxygen species (ROS) in not only angiotensin II-induced cardiovascular diseases but also the pathophysi-
ology of heart failure.\textsuperscript{12,13} Xanthine oxidoreductase (XOR)\textsuperscript{14} and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase\textsuperscript{15} are known to be the major enzymes generating ROS (superoxide). Either XOR\textsuperscript{13,16–18} or NADPH oxidase\textsuperscript{15,19,20} is reported to be responsible for various experimental cardiovascular diseases and to be increased in human heart failure.\textsuperscript{19,21,22} However, the potential contribution of XOR and NADPH oxidase to diastolic heart failure or their relative role in heart failure is undefined.

In the present study, using DS rats with overt diastolic heart failure, we examined whether ARB can reverse established diastolic heart failure and also examined the potential role of XOR and NADPH oxidase in diastolic heart failure. We have obtained the evidence that ARB ameliorates diastolic heart failure in DS rats, being mediated by XOR rather than NADPH oxidase.

Materials and Methods

Animals

All of the procedures were in accordance with institutional guidelines for animal research. DS rats (Japan SLC Inc, Shizuoka, Japan) were used in the present study. At 7 weeks of age, the diet of DS rats was switched from a 0.3% NaCl (low-salt) to an 8% NaCl (high-salt) diet. Control DS rats were fed a 0.3% NaCl diet throughout the experiments.

Therapeutic Effect of Candesartan, Hydralazine, Apocynin, and Oxypurinol on DS Rats With Overt Diastolic Heart Failure

Previously, we\textsuperscript{7,10} and other groups of investigators\textsuperscript{6,9} have shown that DS rats, fed high-salt diet from 7 weeks of age, develop progressive hypertension and exhibit diastolic heart failure at 20 weeks of age. As shown in Figure S1 (available online at http://hyper.ahajournals.org), 7-week-old DS rats began to be fed a high-salt diet and then were subjected to echocardiography at 20 weeks of age to confirm the onset of diastolic heart failure. Twenty-week–old DS rats with overt diastolic heart failure, confirmed by echocardiography, were randomly assigned to 5 groups, including vehicle (0.5% carboxymethyl cellulose), candesartan (1 mg/kg per day), hydralazine (20 mg/kg per day), apocynin (0.3 mmol/kg per day), and oxypurinol (40 mg/kg per day), and drug treatment was performed for 4 weeks (until 24 weeks of age). Candesartan was suspended in 0.5% carboxymethyl cellulose and given to rats as the drinking water. The concentration of apocynin and oxypurinol in the drinking water was 3 and 1 mmol/L, respectively. Blood pressure was measured with the tail-cuff method every week, and echocardiographic assessment was performed biweekly during drug treatment (Figure S1). During 4 weeks of drug treatment, animals were carefully monitored, the symptoms of heart failure were carefully checked, and the number of dead rats was recorded every day to examine survival rate. At the end of 4 weeks of drug treatment, surviving 24-week–old DS rats in each group were anesthetized with ether, blood was collected by cardiac puncture, and the heart, lung, and liver were rapidly excised from each rat to examine cardiac hypertrophy, inflammation, remodeling, and various biochemical and molecular parameters related to oxidative stress.

The detailed methods are described in the online supplemental data.

Results

Characteristics of 20-Week-Old Salt-Loaded DS Rats With Established Diastolic Heart Failure

As shown in Table S1 and Figure S2, 20-week-old salt-loaded DS rats were characterized by prominent LV hypertrophy, marked LV relaxation abnormality, LV chamber dilatation, and marked pulmonary edema and hepatic congestion, but by the preserved LV systolic function, findings confirmed that DS rats used in this work exhibited established diastolic heart failure. Furthermore, DS rats with overt diastolic heart failure were also characterized by marked LV inflammation, interstitial fibrosis, and coronary remodeling, as shown by histological examination, and characterized by the significant decrease in Ser\textsuperscript{16-kinase phosphorylated phospholamban protein levels relative to the same age of control DS rats fed a low-salt diet (Table S1 and Figure S2). These results are in good agreement with previous reports by us\textsuperscript{7,10} and others\textsuperscript{6,11}.

Cardiac NADPH Oxidase, XOR, ROS, and Mitogen-Activated Protein Kinase

LV NADPH oxidase activity, XOR activity, and superoxide levels in salt-loaded DS rats with overt heart failure were 3.0-fold, 2.2-fold, and 2.4-fold higher, respectively, than those in low-salt–fed control DS rats (Figure S3A through S3C). LV phospho-ASK1 and phospho-extracellular signal regulated kinase (ERK) levels in salt-loaded DS rats were increased by 1.6-fold and 1.8-fold, respectively, compared with those in control DS rats (Figure S3D and S3E).

Effect of Drug Treatment on Blood Pressure and Survival of DS Rats With Overt Heart Failure

As shown in Figure S4, candesartan, hydralazine, and apocynin at the dose used in this work significantly reduced blood pressure of DS rats with overt heart failure to a comparable degree throughout 4 weeks of the treatment (from 20 to 24 weeks of age). On the other hand, oxypurinol did not significantly lower blood pressure of DS rats throughout the treatment.

Figure 1 indicates Kaplan–Meier survival curves of DS rats treated with vehicle or each drug from 20 to 24 weeks of age. Survival rate of DS rats at the end of drug treatment (24 weeks of age) was significantly higher in the candesartan (Can), hydralazine (Hyd), and apocynin (Apo) groups than in the vehicle (Veh) group (Figure 1).
weeks of age) was 7% for vehicle, 86% for candesartan, 40% for hydralazine, 36% for apocynin, and 57% for oxypurinol. Candesartan prolonged the survival rate of DS rats more than vehicle (P<0.01) and hydralazine (P<0.01). Oxypurinol also prolonged the survival rate of DS rats more than vehicle (P<0.01). Apocynin and hydralazine tended to improve survival of DS rats compared with vehicle, although there was no statistically significant difference.

Effect of Drug Treatment on Cardiac Function and Lung and Liver Weights
As shown by E/A (ratio of peak early diastolic filling velocity to peak velocity at atrial contraction) during drug treatment in Figure 2A, only candesartan significantly reversed cardiac diastolic dysfunction (P<0.01). Hydralazine failed to prevent the deterioration of LV chamber dilatation, whereas candesartan, apocynin, and oxypurinol significantly prevented the progression of LV chamber dilatation relative to hydralazine (Figure 2B). LV systolic function was normal in DS rats with established diastolic heart failure (Table S1) and was not affected by each drug throughout the treatment (data not shown).

After completion of 4 weeks of each drug treatment, lung and liver weights of surviving 24-week-old DS rats (n=6 in hydralazine treatment, 12 in candesartan, 5 in apocynin, and 8 in oxypurinol) were measured and compared with those of 20-week-old DS rats before drug treatment. As shown in Figure S5A, surviving 24-week-old DS rats treated with candesartan had similar lung weight to 20-week-old DS rats before drug treatment, whereas surviving DS rats treated with hydralazine (P<0.01), apocynin (P<0.01), or oxypurinol (P<0.05) had larger lung weight than 20-week-old DS rats. However, lung weight of DS rats treated with apocynin (P<0.05) or oxypurinol (P<0.01) was significantly smaller than that of hydralazine-treated DS rats. As shown in Figure SSB, the increase in liver weight of DS rats was not prevented by 4 weeks of hydralazine treatment (P<0.01) but prevented by candesartan, apocynin, and oxypurinol.

LV Weight, Macrophage Infiltration, Interstitial Fibrosis, Coronary Arterial Remodeling, and Ser16-Phosphorylated Phospholamban
After completion of 4 weeks of drug treatment, surviving 24-week-old DS rats in each group and 20-week-old DS rats before drug treatment were compared in detail. As shown in Figure 3A, candesartan significantly regressed LV hypertrophy (P<0.01) in DS rats with overt heart failure, whereas hydralazine, apocynin, or oxypurinol did not regress LV hypertrophy in DS rats. As shown in Figure 3B and 3C, candesartan and oxypurinol, but not hydralazine or apocynin, significantly reversed LV macrophage infiltration (P<0.01) and interstitial fibrosis (P<0.01). Apocynin treatment led to less LV macrophage infiltration (P<0.05) and less interstitial fibrosis (P<0.05) than hydralazine treatment. Either candesartan or oxypurinol significantly reduced LV TUNEL-positive nuclei of DS rats, but hydralazine or apocynin did not reduce it (Figure S6A). Compared with 20 weeks of age, coronary arterial thickening was further augmented in 24-week-old DS rats treated with hydralazine (P<0.01), apocynin (P<0.01), or oxypurinol (P<0.01), whereas it was not increased in DS rats treated with candesartan (Figure S6B). As mentioned above, 20-week-old DS rats exhibited the downregulation of LV Ser16-phosphorylated phospholamban (Table S1 and Figure S2). However, candesartan and oxypurinol significantly augmented LV Ser16-phosphorylated phospholamban, whereas apocynin or hydralazine did not affect it (Figure S6C).

LV NADPH Oxidase, XOR, ROS, ASK1, and ERK
As shown in Figure 4 and Figure S7, LV NADPH oxidase activity, XOR activity, superoxide, and phosphorylation of ASK1 and ERK in 24-week-old surviving hydralazine-treated DS rats did not differ from those in 20-week-old DS rats. On the other hand, candesartan significantly attenuated LV NADPH oxidase activity (P<0.01), XOR activity (P<0.01), and superoxide (P<0.01) of DS rats (Figure 4), being accompanied by the significant attenuation of phosphorylation of LV ASK1 (P<0.01) and ERK (P<0.01; Figure S7).
Oxypurinol did not significantly inhibit LV NADPH oxidase activity but significantly attenuated LV XOR activity (P<0.01), which was associated with the reduction of LV superoxide (P<0.01) and phosphorylation of ASK1 (P<0.01) and ERK (P<0.01). Apocynin significantly attenuated LV NADPH oxidase activity (P<0.01) of DS rats but did not reduce LV XOR activity, LV superoxide levels, or phosphorylation of ASK1 and ERK.

Discussion

The main purpose of our present work was to examine the effect of candesartan on established diastolic heart failure, focusing on the roles of XOR and NADPH oxidase. The major finding of this work is that cardiac XOR plays a critical role in the beneficial effect of candesartan on established hypertensive diastolic heart failure. Thus, our present work provided a novel mechanism underlying the exacerbation of diastolic heart failure and the beneficial effect of candesartan on hypertensive diastolic heart failure.

In this work, we examined whether candesartan can reverse established diastolic heart failure in DS rats by comparing candesartan-treated DS rats with DS rats before drug treatment. Interestingly, initiation of candesartan treatment at the established diastolic heart failure of DS rats reversed cardiac hypertrophy, inflammation, and fibrosis; normalized cardiac Ser16-phosphorylated phospholamban levels; restored cardiac relaxation abnormality; and prolonged survival rate. On the other hand, treatment of DS rats with hydralazine, which exerted similar hypotensive effects to candesartan throughout the treatment, did not significantly improve cardiac hypertrophy, remodeling, Ser16-phosphorylated phospholamban levels, and diastolic dysfunction and did not prolong survival rate. These observations provide the evidence that candesartan restored diastolic heart failure in DS rats by inducing reverse cardiac remodeling, independent of blood pressure.

Accumulating evidence indicate that ROS is importantly implicated in angiotensin II–induced cardiac remodeling by stimulating cardiac hypertrophy, inflammation, and fibrosis.
However, the precise role of ROS in the improvement of heart failure, particularly diastolic heart failure, by ARB is not defined. Therefore, in the present work, we examined the effect of candesartan on cardiac ROS in DS rats with diastolic heart failure. We also examined the effect of candesartan on cardiac ASK1 and ERK, because these protein kinases are the main intracellular signaling molecules activated by ROS and they play a key role in the development of cardiac hypertrophy and remodeling, as reported by us and others. Of note, candesartan significantly attenuated LV superoxide and the phosphorylation of LV ASK1 and ERK in DS rats. On the other hand, despite a similar hypotensive effect between candesartan and hydralazine, hydralazine did not improve LV superoxide, ASK1, or ERK in DS rats. Together, these results support the notion that the reversal of cardiac remodeling by candesartan might be at least in part attributed to the inhibition of ASK1 and ERK secondary to the attenuation of ROS.

XOR and NADPH oxidase are well known to be the major enzymes generating ROS. However, there is no report on the role of these ROS-generating enzymes in the exacerbation of diastolic heart failure. In the present study, we found that DS rats with diastolic heart failure were characterized by the increase in both cardiac XOR and NADPH oxidase activities (Figure S3). Interestingly, these characteristics of DS rats differ from those of hypertensive systolic heart failure rats, which exhibit the increased cardiac XOR activity without the increase in cardiac NADPH oxidase activity. To examine the potential contribution of angiotensin II receptor subtype 1 to the enhancement of XOR and NADPH oxidase activities in DS rats at established diastolic heart failure, we examined the impact of candesartan on these enzymes in DS rats. We found that candesartan significantly attenuated the increase in both cardiac NADPH and XOR activities. These observations provide the evidence for the direct involvement of angiotensin II receptor subtype 1 in the augmentation of both XOR and NADPH oxidase activities in DS rats at established diastolic heart failure.

Cardiac XOR and NADPH oxidase activities are reported to be increased in various experimental models of cardiac diseases and to be involved in cardiac hypertrophy and remodeling. Furthermore, the activities of XOR and NADPH oxidase are enhanced in patients with heart failure. However, the relative role of XOR and NADPH oxidase in the pathophysiology of established heart failure is poorly understood. The potential contribution of these ROS-generating enzymes to the beneficial effects of ARB on diastolic heart failure remains to be determined. Furthermore, to the best of our knowledge, there is no report concerning the direct comparison between the pharmacological inhibition of NADPH oxidase and XOR regarding the therapeutic effect on diastolic heart failure. Therefore, in this work, we directly compared the effect of oxypurinol (a specific XOR inhibitor) and apocynin (a specific NADPH oxidase inhibitor) on established diastolic heart failure in DS rats. Blood pressure of DS rats with established heart failure was significantly lowered by apocynin to a comparable degree to candesartan and hydralazine throughout the treatment, whereas blood pressure was not reduced by oxypurinol. These results reveal that NADPH oxidase, but not XOR, is responsible for hypertension in DS rats at established diastolic heart failure. Of note are the observations that, despite no lowering of blood pressure, oxypurinol, with the significant inhibition of LV XOR activity, prevented the exacerbation of LV chamber dilatation, reversed LV inflammation and fibrosis, upregulated Ser-phosphorylated phospholamban levels, and significantly prolonged survival of DS rats. Furthermore, as in the case of candesartan treatment, the inhibition of XOR activity with oxypurinol significantly attenuated LV superoxide levels and phosphorylated ASK1 and ERK in DS rats. These observations, taken together with the present findings that candesartan significantly reduced cardiac XOR activity, demonstrate that cardiac XOR is involved in the restoration of diastolic heart failure by candesartan in DS rats.

Treatment of DS rats with apocynin inhibited cardiac NADPH oxidase activity at least to a comparable degree to candesartan (Figure 4A). However, apocynin failed to reduce LV superoxide of DS rats, did not reverse LV inflammation and fibrosis, did not upregulate Ser-phosphorylated phospholamban, and did not prolong survival of DS rats. Therefore, differing from XOR, NADPH oxidase seems to play a minor role in the beneficial effect of candesartan on diastolic heart failure in DS rats. However, apocynin treatment exerted less LV chamber dilatation, less macrophage infiltration, and less cardiac fibrosis than hydralazine treatment, indicating that NADPH oxidase also plays some role in the exacerbation of diastolic heart failure in DS rats. Moreover, in the present study, we did not examine the effect of a higher dose of apocynin on established diastolic heart failure. Therefore, it cannot be completely excluded that the therapeutic effect of candesartan on diastolic heart failure might be attributed to the cumulative inhibition of XOR and NADPH oxidase. Further study is needed to elucidate the precise role of NADPH oxidase in the exacerbation of hypertensive diastolic heart failure.

In conclusion, candesartan slowed the exacerbation of hypertensive diastolic heart failure in DS rats by causing reverse cardiac remodeling. Cardiac XOR plays a critical role in the reversal of hypertensive diastolic heart failure in DS rats by candesartan. Our present work provides the novel insight into the mechanism responsible for the beneficial effect of candesartan on hypertensive diastolic heart failure.

Perspectives
Despite a large number of reports on the mechanism of systolic heart failure, the mechanism and the therapeutic strategy of hypertensive diastolic heart failure are poorly understood. Although blockers of the renin-angiotensin system, such as ARB and angiotensin-converting enzyme inhibitors, are established to be the useful therapeutic agents for systolic heart failure, the clinical use of the renin-angiotensin system blockers for hypertensive diastolic heart failure remains to be defined. Therefore, the experimental study on the therapeutic effect of ARB on established diastolic heart failure is of great clinical relevance. Furthermore, NADPH oxidase and XOR are major enzymes generating ROS and are supposed to be responsible for cardiovascular diseases. However, the relative contribution of NADPH oxidase and XOR in established...
heart failure, particularly diastolic heart failure, remains to be defined. Therefore, our present findings provide the new insight into not only the role of angiotensin II and ROS in hypertensive diastolic heart failure but also the mechanism underlying the exacerbation of diastolic heart failure.

Sources of Funding
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Disclosures
None.

References
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Online Materials and Methods

Drugs

Candesartan was kindly gifted from Takeda Pharmaceutical Co., Ltd (Osaka, Japan). Hydralazine and apocynin, a specific NADPH oxidase inhibitor\(^1\), were purchased from Sigma-Aldrich Co. (Missouri, USA). Oxypurinol, a specific XOR inhibitor, was from Sigma Medical Co., Ltd (Osaka, Japan). Twenty-week-old DS rats with overt diastolic heart failure, confirmed by echocardiography, were randomly assigned to 5 groups, including (1) vehicle (0.5 % carboxymethyl cellulose [CMC]), (2) candesartan (1 mg/kg/day), (3) hydralazine (20 mg/kg/day), (4) apocynin (0.3 mmol/kg/day), and (5) oxypurinol (40 mg/kg/day), and drug treatment was performed for 4 weeks (until 24 weeks of age). The concentration of apocynin and oxypurinol in the drinking water was 3 mmol/L and 1 mmol/L, respectively. The dose of apocynin\(^2-5\) and oxypurinol\(^6\) was determined, according to the previous reports.

Echocardiographic assessment

Transthoracic echocardiographic studies were performed with an echocardiographic system equipped with 12-MHz echocardiographic probe (PHILIPS SONOS-4500) as previously described in detail\(^7\). In brief, DS rats were lightly anesthetized with intraperitoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and were held in the half left-lateral position. M-mode tracings were recorded through left ventricular (LV) anterior and posterior walls (AW and PW, respectively) at the papillary muscle level to measure left ventricular end-diastolic dimension, left ventricular end-systolic dimension, fractional shortening, left ventricular ejection fraction, left ventricular anterior wall thickness at end diastole, and posterior wall thickness at end diastole. Pulse-wave Doppler spectra (E and A wave velocity) of mitral inflow were recorded from

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\(^1\) Apocynin is a selective NADPH oxidase inhibitor.
\(^2\) Oxypurinol is a specific XOR inhibitor.
\(^3\) Apocynin was used at 2-5 mmol/L for various studies.
\(^4\) Oxypurinol was used at 1 mmol/L for various studies.
the apical 4-chamber view, with the sample volume placed near the tips of the mitral leaflets and adjusted to the position at which velocity was maximal and the flow pattern laminar.

**Cardiac NADPH oxidase activity**

Left ventricular tissues were homogenized with an Ultraturrax T8, centrifuged, and NADPH oxidase activity of the resulting supernatant was measured by lucigenin chemiluminescence in the presence of 10 µM NADPH and 10 µM lucigenin as electron acceptor, as described\(^8\). Protein concentrations were measured by the method of Bradford.

**Cardiac xanthine oxidoreductase activity**

XOR activity was measured using the horseradish peroxidase-linked Amplex Red fluorescence assay kit (Molecular Probes, Invitrogen Detection Technologies), according to the manufacturer’s instruction. In brief, left ventricular tissues were homogenized in cell lysis buffer (Cell Signaling Tech., Beverly, MA) containing 1 mM phenylmethyl sulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), followed by centrifugation. The resulting supernatant was added to a working solution containing Amplex Red reagent (50 µM), xanthine (0.1 mM) and horseradish peroxidase type II (0.1 U/ml), incubated at 37 ºC for 30 minutes, and the H\(_2\)O\(_2\) production was measured in the absence or presence of oxypurinol (1 mM) in order to subtract the background. Fluorescence readings were made in duplicate in a 96-well plate at Ex/Em = 544/590 nm.

**Measurement of cardiac superoxide**

The left ventricular tissue, removed from DS rats, was immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate cardiac superoxide levels in situ, as described in detail\(^9\). DHE fluorescence of the sections was quantified using Lumina Vision version 2.2, analysis software.

**Preparation of cardiac protein extracts and Western blot analysis**
Our detailed method has been described previously. Briefly, after left ventricular protein extracts were subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows: anti-phospho-ASK1, anti-phospho-ERK (x 2000, Cell Signaling TECHNOLOGY), anti-ERK (x 2000, Cell Signaling TECHNOLOGY), anti-eNOS (x 5000, BD Transduction Laboratories), anti-α-tubulin (x 5000, CALBIOCHEM), anti-sarcoplasmatic reticulum Ca^{2+} ATPase (SERCA2a) (x 2000, Affinity BioReagents, Inc.), anti-phospholamban (x 1000, Affinity BioReagents, Inc.), and anti-Ser^{16}-phosphorylated phospholamban (x 1000, Upstate Biotechnology, Inc.). In individual samples, each value was correct for that of α-tubulin.

**Histological examination**

The hearts from DS rats were fixed in 4 % paraformaldehyde overnight. Then, they were embedded in paraffin, sectioned into 5-µm slices, stained with Sirius Red F3BA (0.5 % in saturated aqueous picric acid, Aldrich Chemical Company) for assessment of interstitial fibrosis and coronary arterial thickness, as previously described. Moreover, the sections were immunostained with anti-ED-1 antibody (working dilution 1:500) for identification of monocytes/macrophages. Positive staining was detected using horseradish peroxidase-conjugated secondary antibodies (Nichirei, Japan), by incubating the sections with diaminobenzidine (DAKO). The number of ED-1-positive cells was counted in 4 horizontal LV sections in individual rats; and the average of ED-1 positive cell number was obtained in individual rats, as described. Apoptosis in cardiac section was detected with the TdT-mediated dUTP nick-end labeling (TUNEL) by utilizing in situ Apoptosis Detection Kit (Takara, Shiga Japan).

**Statistical analysis**

All data are presented as mean±SEM. The data on time course experiments were analyzed by
two-way ANOVA, followed by Fisher’s PLSD test, using StatView for Windows (SAS Institute, Inc. Cary, U.S.A.). Comparison between 2 groups was analyzed by unpaired Student’s t-test. In comparison among more than 2 groups, statistical significance was determined with one-way ANOVA, followed by Fisher’s PLSD test. Survival was analyzed by the standard Kaplan-Meier analysis with log-rank test and χ² analysis. In all tests, differences were considered statistically significant at a value of P<0.05.
Online References


Online Figure Legends

**Figure S1** Experimental design of this work

DS rats were fed high-salt diet from 7 weeks of age, were subjected to echocardiography at 20 weeks of age to confirm the onset of diastolic heart failure, and then were treated with each drug from 20 to 24 weeks of age. After termination of 4 weeks of drug treatment, each group of surviving 24-week-old DS rats were compared in detail, regarding cardiovascular injury and oxidative stress-related parameters.

**Figure S2** Representative photographs of echocardiography (A), heart, lung, and liver (B), LV histological findings (C), and western blot analysis (D) in 20-week-old DS rats fed high-salt diet and low-salt diet

Abbreviations: Low, 20-week-old DS rats fed low-salt diet; High, 20-week-old DS rats fed high-salt diet from 7 weeks of age

**Figure S3** LV NADPH oxidase activity (A), XOR activity (B), superoxide (C), phospho-ASK1 (D), and phospho-ERK (E) of salt-loaded 20-week-old DS rats with overt heart failure, relative to the same age of DS rats fed low-salt diet

Abbreviations: Low, 20-week-old DS rats fed low-salt diet; High, 20-week-old DS rats fed high-salt diet from 7 weeks of age. Values are mean±SEM (n=4-9).

**Figure S4** Blood pressure of DS rats with overt heart failure during 4 weeks of drug treatment (from 20 to 24 weeks of age)

Abbreviations: Veh, vehicle (n=14); Can, candesartan (n=14); Hyd, hydralazine (n=15); Apo, apocynin (n=14); Oxy, oxypurinol (n=14). Values are mean±SEM.

**Figure S5** Lung weight (A) and liver weight (B) of surviving DS rats subjected to 4 weeks of drug treatment

Abbreviations are the same as in Figure S4. Pre indicates 20-week-old DS rats with overt
heart failure, not subjected to drug treatment. Lung and liver weights were determined in surviving 24-week-old DS rats subjected to 4 weeks of drug treatment. Each value in (A) and (B) represents mean±SEM (n= 5 in Pre, 6 in Hyd, 12 in Can, 5 in Apo, 8 in Oxy).

**Figure S6** LV apoptosis (A), coronary arterial thickening (B), and Ser$^{16}$-phosphorylated phospholamban expression (C) of surviving DS rats subjected to 4 weeks of drug treatment. Abbreviations are the same as in Figure S4. Pre indicates 20-week-old DS rats with overt heart failure, not subjected to drug treatment. Each value represents mean±SEM (n= 5 in Pre, 6 in Hyd, 12 in Can, 5 in Apo, 8 in Oxy).

**Figure S7** LV phospho-ASK1 (A) and phospho-ERK (B) of surviving DS rats subjected to 4 weeks of drug treatment. Abbreviations are the same as in Figure S4. Pre indicates 20-week-old DS rats with overt heart failure, not subjected to drug treatment. Each value represents mean±SEM (n= 5 in Pre, 6 in Hyd, 12 in Can, 5 in Apo, 8 in Oxy).
### Table S1. Characteristics of 20-week-old Dahl salt sensitive hypertensive rats fed high Na diet from 7 weeks of age, relative to those fed low Na diet

<table>
<thead>
<tr>
<th>Characteristics of cardiac remodeling</th>
<th>Low Na</th>
<th>High Na</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>430±5</td>
<td>360±13</td>
<td>P&lt;0.01</td>
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<td>Blood pressure (mmHg)</td>
<td>134±4</td>
<td>217±7</td>
<td>P&lt;0.01</td>
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<td>Echocardiography</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>388±16</td>
<td>449±11</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>LVAd (mm)</td>
<td>1.47±0.02</td>
<td>2.27±0.02</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>LVPw (mm)</td>
<td>1.48±0.01</td>
<td>2.28±0.02</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>LVDd/BW (mm/kg BW)</td>
<td>21.0±0.3</td>
<td>24.9±1.0</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>LVDs/BW (mm/kg BW)</td>
<td>13.9±0.3</td>
<td>16.3±0.8</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>EF (%)</td>
<td>68±1</td>
<td>69±1</td>
<td>NS</td>
</tr>
<tr>
<td>FS (%)</td>
<td>34±1</td>
<td>34±1</td>
<td>NS</td>
</tr>
<tr>
<td>E/A</td>
<td>1.9±0.2</td>
<td>4.5±0.5</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>LV weight/BW (mg/g BW)</td>
<td>2.3±0.0</td>
<td>4.8±0.2</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Lung weight/BW (mg/g BW)</td>
<td>3.4±0.1</td>
<td>5.1±0.2</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Liver weight/BW (mg/g BW)</td>
<td>33.8±0.7</td>
<td>38.1±1.5</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Cardiac histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED-1 positive cell (counts/mm²)</td>
<td>23.6±2.5</td>
<td>77.0±5.1</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Collagen volume fraction (%)</td>
<td>3.3±0.2</td>
<td>7.8±0.3</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Coronary arterial thickening (%)</td>
<td>27±2</td>
<td>47±3</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>TUNEL-positive nuclei (counts/mm²)</td>
<td>0.6±0.3</td>
<td>2.5±0.3</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Cardiac calcium regulatory proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERCA2a/tublin</td>
<td>1.00±0.04</td>
<td>0.97±0.04</td>
<td>NS</td>
</tr>
<tr>
<td>phospholamban/tublin</td>
<td>1.00±0.07</td>
<td>0.99±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>P-phospholamban/tublin</td>
<td>1.00±0.06</td>
<td>0.73±0.02</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

High Na indicates 20-week-old DS rats fed high Na diet from 7 weeks of age, whereas Low Na indicates the same age of DS rats fed low Na diet. BW, body weight; LV, left ventricular; LVAd, left ventricular anterior wall thickness; LVPw, left ventricular posterior wall thickness; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; EF, ejection fraction; FS, fractional shortening; E/A, ratio of peak early diastolic filling velocity to peak velocity at atrial contraction; SERCA2a, sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase2a; P-phospholamban, Ser¹⁶-phosphorylated phospholamban. Values are mean±SEM (n=5-13).
Figure S2

(A) M mode

Low

High

E/A

(L) Low (H) High

1 cm

Heart

Lung

Liver

(C) ED-1 positive cell

Low

High

Interstitial collagen

Coronary artery

(D) SERCA2a

Low

High

phospholamban

p-phospholamban

1 cm
Figure S3

(A) NADPH oxidase activity (x10² CPM/mg)

(B) XOR activity (mUnit/mg)

(C) Superoxide (%)

(D) p-ASK1/tubulin

(E) p-ERK/tubulin

P<0.01
Figure S4

Age (weeks)

-mmHg-

Blood pressure

* P<0.01 vs Veh

Veh  Can  Oxy
Hyd  Apo
Figure S5

# P<0.05, *P<0.01 vs Pre
+ P<0.05, †P<0.01 vs Hyd

(A) Lung weight (mg/g BW)

(B) Liver weight (mg/g BW)
Figure S6

# P<0.05, *P<0.01 vs Pre
+ P<0.05, †P<0.01 vs Hyd

(A) TUNEL positive nuclei (counts/mm²)

(B) Coronary arterial thickening (%)

(C) ß-phospholamban/tubulin
Figure S7

# P<0.05, *P<0.01 vs Pre
† P<0.05, †† P<0.01 vs Hyd

(A) (B)