Novel Mechanism and Role of Angiotensin II–Induced Vascular Endothelial Injury in Hypertensive Diastolic Heart Failure

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Objective—The mechanism and role of angiotensin II–induced vascular endothelial injury is unclear. We examined the molecular mechanism of angiotensin (AII)-induced vascular endothelial injury and its significance for hypertensive diastolic heart failure.

Methods and Results—We compared the effect of valsartan and amlodipine on Dahl salt-sensitive hypertensive rats (DS rats). Valsartan improved vascular endothelial dysfunction of DS rats more than amlodipine, by inhibiting endothelial apoptosis and eNOS uncoupling more. Moreover, valsartan inhibited vascular apoptosis signal-regulating kinase 1 (ASK1) more than amlodipine. Thus, AT1 receptor contributed to vascular endothelial apoptosis, eNOS uncoupling, and ASK1 activation of DS rats. Using ASK1−/− mice, we examined the causative role of ASK1 in endothelial apoptosis and eNOS uncoupling. All infusion in wild-type mice markedly caused vascular endothelial apoptosis and eNOS uncoupling accompanied by vascular endothelial dysfunction, whereas these effects of AII were absent in ASK1−/− mice. Therefore, ASK1 participated in AII-induced vascular endothelial apoptosis and eNOS uncoupling. Using tetrahydrobiopterin, we found that eNOS uncoupling was involved in vascular endothelial dysfunction in DS rats with established diastolic heart failure.

Conclusion—AII-induced vascular endothelial apoptosis and eNOS uncoupling were mediated by ASK1 and contributed to vascular injury in diastolic heart failure of salt-sensitive hypertension.

Key Words: angiotensin ■ endothelium ■ heart failure ■ nitric oxide ■ signal transduction

Salt-sensitive hypertensive patients are more prone to cardiovascular diseases than their salt-insensitive counterparts. Therefore, it is a clinically important issue to determine the mechanism and the therapeutic strategy of cardiovascular diseases in salt-sensitive hypertension. Vascular endothelial function plays a key role in the pathophysiology and the prognosis of cardiovascular diseases, including atherosclerosis, ischemic heart disease, and heart failure. However, the detailed molecular mechanisms and the pathological significance of vascular endothelial dysfunction in salt-sensitive hypertension are unknown.

Apoptosis signal-regulating kinase 1 (ASK1), a mitogen-activated protein kinase kinase, has been identified as a proapoptotic signaling molecule. ASK1 is activated in response to a variety of stress stimuli, such as reactive oxygen species (ROS), angiotensin II (AII), or cytokines, etc. Accumulating in vitro evidence indicates that ASK1 participates in not only apoptosis but also various cellular responses, including cell differentiation and growth, or gene expression. Previously, we have shown that ASK1 is responsible for cardiac hypertrophy and fibrosis, vascular intimal hyperplasia, and ischemia-induced angiogenesis. Furthermore, other investigators have also reported that ASK1 is implicated in cardiac myocyte death and remodeling induced by ischemia. However, the role of ASK1 in vascular endothelial injury is unclear.

In the present study, by using Dahl salt-sensitive hypertensive rats, the useful model of not only salt-sensitive hypertension but also diastolic heart failure, and ASK1-deficient mice, we have obtained the first evidence that AII-induced vascular endothelial apoptosis and eNOS uncoupling are mediated by the activation of ASK1 and play a key role in exacerbation of vascular injury in salt-sensitive hypertensive rats at the stage of diastolic heart failure.

Materials and Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Dahl salt-sensitive hypertensive rats (DS rats) (Japan SLC Inc, Shizuoka, Japan) were used in the present study.
Male AKS1−/− mice and wild-type mice (C57BL/6J) were used in the present study.

Comparative Effect of Valsartan and Amlodipine on Vascular Injury and Survival Rate of DS Rats Fed High-Salt Diet
To elucidate the direct role of angiotensin II (AII) in vascular diseases of salt-sensitive hypertension, we compared the effect of valsartan (Novartis) and amiodipine (Pfizer) on DS rats. Twelve-week-old DS rats, which had fed a high-salt diet from 7 weeks of age, were given vehicle (0.5% carboxymethyl cellulose [CMC]), valsartan (10 mg/kg/d), or amiodipine (1 mg/kg/d), by gastric gavage once a day for 4 weeks (until 16 weeks of age).

In separate experiments, 12-week-old DS rats, fed a high-salt diet from 7 weeks of age, were orally given vehicle, valsartan (10 mg/kg/d), or amiodipine (1 mg/kg/d), in the same manner as the above experiment, and survival rate was examined until 24 weeks of age.

Effect of Angiotensin II Infusion on Wild-type and ASK1−/− Mice
To examine the role of ASK1 in vascular endothelial injury by angiotensin II (AII), we compared the effects of chronic AII infusion on wild-type and ASK1−/− mice. All (600 ng/kg/min) was subcutaneously infused to mice via osmotic minipump (ALZA CO) for 4 weeks.

Role of eNOS Uncoupling in End-Stage Heart Failure of DS Rats
Using 20-week-old DS rats with overt heart failure, we examined the role of eNOS uncoupling in end-stage heart failure of DS rats. Tetrahydrobiopterin (BH4); sapropterin hydrochloride, Daiichi Suntory Pharma Co Ltd, Tokyo; 10 mg/kg/d), apocynin (0.3 mmol/kg/d), or hydralazine (20 mg/kg/d) was orally given to 20-week-old DS rats with overt heart failure, for 4 weeks (until 24 weeks of age). Furthermore, as a control, we also examined the effect of tetrahydronocerperin (H11546; Schircks Laboratories) (10 mg/kg/d), which has similar antioxidant properties to BH4 but is not directly linked to eNOS coupling and activity, in DS rats.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Effects of Valsartan and Amlodipine on Vascular Endothelial Function and Remodeling, and Survival of DS Rats
Valsartan and amiodipine slightly and comparably reduced blood pressure of DS rats, throughout 4 weeks of drug treatment (supplemental Figure IA, available online at http://atvb.ahajournals.org). Vascular endothelium-dependent relaxation to acetylcholine of 16-week-old salt-loaded DS rats was remarkably impaired, compared with control DS rats (P<0.01) (supplemental Figure IB), whereas vascular endothelium-independent relaxation by sodium nitroprusside did not differ between the 2 groups of DS rats (data not shown). Despite comparable blood pressure lowering between valsartan and amiodipine, valsartan more potently improved vascular endothelium-dependent relaxation than amiodipine (P<0.05; supplemental Figure IB). Valsartan significantly suppressed coronary arterial thickening, compared with vehicle (P<0.05), whereas amiodipine did not significantly suppress it (supplemental Figure IC).

As shown in supplemental Figure ID, valsartan treatment significantly prolonged survival rate of DS rats, compared with vehicle (P<0.01), whereas amiodipine did not prolong it.

Effects of Valsartan and Amlodipine on Vascular Endothelial Apoptosis of DS Rats
As shown in Figure 1, vascular endothelial apoptosis and ASK1 phosphorylation were significantly enhanced in salt-loaded DS rats. Valsartan attenuated vascular endothelial apoptosis of salt-loaded DS rats more than amiodipine (P<0.05), which was associated with more inhibition of ASK1 by valsartan than amiodipine (P<0.05).

Effects of Valsartan and Amlodipine on Vascular NADPH Oxidase and Superoxide of DS Rats
As shown in supplemental Figure II, 16-week-old salt-loaded DS rats had more NADPH oxidase activity (P<0.01) and
vascular superoxide (P<0.01) detected with the fluorescent probe dihydroethidium, than the age-matched control DS rats. The results on preincubation of vascular sections with polyethylene glycol (PEG)-SOD or Tiron confirmed that the increased vascular dihydroethidium fluorescence in salt-loaded DS rats indeed represented superoxide itself (supplemental Figure III). Treatment with valsartan or amlodipine from 12 to 16 weeks of age significantly reduced all the above mentioned parameters of DS rats. However, vascular superoxide levels were reduced by valsartan more than amlodipine (P<0.05; supplemental Figure IIB).

Effects of Valsartan and Amlodipine on Vascular eNOS of DS Rats
As shown in Figure 2, the ratio of dimer to monomer of eNOS, eNOS activity, and plasma NOx levels in 16-week-old salt-loaded DS rats were reduced to 27% (P<0.01), 47% (P<0.01), and 37% (P<0.01) in DS rats, respectively, compared with control DS rats. As shown in supplemental Table I, vascular BH4 levels in salt-loaded DS rats were lower than those in control DS rats (P<0.01), whereas vascular oxidized biotin levels in salt-loaded DS rats were higher than those in control DS rats (P<0.05). As shown in supplemental Figure IV, using lucigenin chemiluminescence, we found that pretreatment of vascular segments with L-NAME significantly decreased superoxide production in salt-loaded DS rats, but not in control salt-unloaded DS rats, indicating that vascular superoxide in salt-loaded DS rats was at least partially derived from eNOS uncoupling. Valsartan prevented the decrease in the ratio of dimer to monomer of eNOS (P<0.01) and the decrease in eNOS activity (P<0.05) in DS rats more than amlodipine (Figure 2). Valsartan significantly prevented the reduction of plasma NOx in DS rats (P<0.01), whereas amlodipine did not.

Effects of Angiotensin II Infusion on Blood Pressure, Vascular ASK1, and Vascular Endothelial Function of ASK1−/− Mice
As shown in supplemental Figure VA, blood pressure elevation in ASK1−/− mice by AII infusion was comparable to that in wild-type mice, throughout 4 weeks of the infusion. As shown in supplemental Figure VB, AII infusion increased the phosphorylation of vascular ASK1×1.7-fold (P<0.01). On the other hand, as expected, ASK1 band was not detected in ASK1−/− mice with or without AII infusion. As shown in Figure 3, there was no significant difference in the dose-response curve of vascular endothelium-dependent relaxation to acetylcholine between wild-type and ASK1−/− mice without AII infusion. However, AII infusion in wild-type mice significantly impaired vascular endothelium-dependent relaxation to acetylcholine (P<0.01). On the other hand, AII infusion in ASK1−/− mice did not at all impair vascular endothelium-dependent relaxation to acetylcholine. Vascular endothelium-independent relaxation by sodium nitroprusside did not differ between wild-type and ASK1−/− mice, regardless of AII infusion (data not shown).

Effects of Angiotensin II Infusion on Vascular NADPH Oxidase Activity, p22phox, and Superoxide of ASK1−/− Mice
As shown in supplemental Figure VIA and VIB, AII infusion in wild-type mice did not at all impair vascular endothelium-dependent relaxation to acetylcholine (P<0.01). On the other hand, as expected, ASK1−/− mice did not at all impair vascular endothelium-dependent relaxation to acetylcholine. Vascular endothelium-independent relaxation by sodium nitroprusside did not differ between wild-type and ASK1−/− mice, regardless of AII infusion (data not shown).

Effect of Angiotensin II Infusion on Vascular Endothelial Apoptosis and eNOS of ASK1−/− Mice
As shown in Figure 4A, AII infusion markedly increased vascular endothelial apoptosis in wild-type mice (P<0.01), but not in ASK1−/− mice. All infusion in wild-type mice significantly reduced the ratio of dimer to monomer of eNOS (P<0.01), whereas AII infusion in ASK1−/− mice did not alter it (Figure 4B). Vascular eNOS activity was reduced by AII infusion in wild-type mice (P<0.05), whereas did not change by AII infusion in ASK1−/− mice (Figure 4C). Vascular phospho-eNOS and total eNOS levels were not...
thickening of surviving 24-week-old DS rats and compared with those of 20-week-old DS rats without drug treatment. As shown in supplemental Figure XA and XB, 20-week-old salt-loaded DS rats exhibited remarkable impairment of vascular endothelial function (P<0.01) and prominent coronary arterial thickening (P<0.01), compared with control DS rats. BH4 significantly reversed vascular endothelial dysfunction of DS rats (P<0.05), whereas apocynin did not reverse it and hydralazine did not prevent further exacerbation of endothelial dysfunction. Furthermore, BH4, but not apocynin or hydralazine, prevented further progression of coronary arterial thickening of DS rats (supplemental Figure XB).

As shown in Figure 6A, BH4 significantly increased the ratio of dimer to monomer of vascular eNOS (P<0.01), compared with 20-week-old DS rats, whereas apocynin or hydralazine treatment did not alter it. Compared with 20-week-old DS rats, vascular eNOS activity was also increased by BH4 (P<0.05) but was not altered by apocynin or hydralazine (Figure 6B). BH4 significantly reduced vascular superoxide levels of DS rats (P<0.05), but apocynin or hydralazine could not alter it (Figure 6C). However, vascular NADPH oxidase activity was significantly decreased by apocynin (P<0.01), but not altered by BH4 (Figure 6D).

Discussion

The major purpose of our work was to examine the mechanism of AII-induced vascular endothelial injury and its role in salt-sensitive hypertensive rats with diastolic heart failure. The major findings were that AII-induced vascular endothelial dysfunction was attributed to ASK1-mediated endothelial apoptosis and eNOS uncoupling, and was involved in vascular injury of hypertensive diastolic heart failure.

We17,20 and others21,22 have reported that AII contributes to not only cardiac hypertrophy and remodeling but also the progression of diastolic heart failure in DS rats. However, the precise role of AII in vascular endothelial injury in DS rats is still unknown. In the present work, to determine the potential role of AII in vascular endothelial injury, we compared the effect of valsartan and amlodipine on vascular injury of DS rats (Figures 1 and 2 and supplemental Figures I and II). Recent report by Julius et al23 on subanalysis of the Valsartan Antihypertensive Long-Term Use Evaluation (VALUE) trial has indicated that valsartan is superior to amlodipine in terms of the prevention of hypertensive heart failure, although its mechanism remains to be clarified. Therefore, our present study, comparing between valsartan and amlodipine in DS rats, is of clinical relevance. In the present work, valsartan more prevented the death of DS rats attributable to heart failure than amlodipine, being associated with greater improvement of vascular endothelial function and coronary arterial remodeling by valsartan. Notably, valsartan suppressed vascular endothelial apoptosis to a greater extent than amlodipine, indicating that the improvement of vascular endothelial dysfunction by valsartan was at least in part mediated by the suppression of endothelial apoptosis. Moreover, valsartan more ameliorated vascular eNOS uncoupling than amlodipine, which was accompanied by more decrease in vascular superoxide and more restoration of eNOS activity by valsartan than by amlodipine (supplemental Figure II and
AS4 Activated in DS Rats in Accordance with the Occurrence of Endothelial Dysfunction. To determine the reason for the absence of vascular endothelial dysfunction in ASK1-deficient mice subjected to AII infusion, we measured vascular endothelial apoptosis, NADPH oxidase, ROS, eNOS uncoupling, and eNOS activity. All infusion markedly caused vascular endothelial apoptosis in wild-type mice, whereas it did not cause apoptosis in ASK1-deficient mice. These observations provide the evidence that ASK1 plays a key role in AII-induced vascular endothelial dysfunction. Furthermore, being consistent with the previous report,24 All infusion significantly induced vascular eNOS uncoupling in wild-type mice, which was accompanied by the significant increase in vascular superoxide and the significant reduction of eNOS activity. On the other hand, vascular eNOS uncoupling and the reduction of eNOS activity did not apparently occur in ASK1-deficient mice infused with AII, and the increase in vascular superoxide by AII infusion was less in ASK1-deficient mice than wild-type mice. All these results, taken together with the findings that AII infusion increased vascular NADPH oxidase activity and p22phox in wild-type and ASK1-deficient mice to a comparable degree and did not affect phospho-eNOS and total eNOS in either strain of mice, provided the evidence that ASK1 is specifically implicated in AII-induced vascular endothelial dysfunction by causing endothelial apoptosis and eNOS uncoupling.

eNOS uncoupling25 and the increase in NADPH oxidase activity26 have been reported in patients with heart failure. Therefore, the investigation on the relative role of eNOS uncoupling and NADPH oxidase in vascular endothelial injury in heart failure is of great clinical relevance. To further elucidate the potential role of eNOS uncoupling in hyperten-

Figure 1. These results show that AII participated in vascular endothelial dysfunction of DS rats, by causing not only endothelial apoptosis but also eNOS uncoupling.

In our current work, we found that vascular ASK1 is activated in DS rats in accordance with the occurrence of endothelial apoptosis and eNOS uncoupling and that AII specifically contributed to ASK1 activation in DS rats, as shown by the significant inhibition of ASK1 by valsartan but not amlodipine (Figure 1). Previously, we have reported that ASK1 is implicated in AII-induced cardiac hypertrophy and fibrosis.12 Thus, ASK1 seems to be an important signaling molecule responsible for cardiovascular diseases. However, the precise role of ASK1 in AII-induced vascular endothelial injury remains to be defined. Therefore, in the present work, by using mice lacking ASK1, we examined the potential role of ASK1 in vascular endothelial dysfunction, apoptosis, and eNOS uncoupling induced by AII (Figures 3 and 4 and supplemental Figures V through VII). Of note are the observations that AII infusion significantly activated vascular ASK1 and significantly impaired vascular endothelial function, whereas AII infusion did not at all impair vascular endothelial function in mice lacking ASK1. These observations provided the first evidence that ASK1 plays a critical role in AII-induced vascular endothelial dysfunction.

Figure 5. Effect of tetrahydrobiopterin, apocynin, and hydralazine on survival rate of DS rats with overt heart failure. Twenty-week-old DS rats with overt heart failure were given vehicle (n = 14), tetrahydrobiopterin (n = 12), apocynin (n = 14), or hydralazine (n = 15) for 4 weeks (until 24 weeks of age), and survival rates of DS rats were compared among each group. Veh indicates vehicle treatment; BH4, tetrahydrobiopterin treatment; Apo, apocynin treatment; Hyd, hydralazine treatment.

Figure 4. Effect of angiotensin II infusion on vascular endothelial apoptosis (A), vascular eNOS uncoupling (B), and eNOS activity (C) of wild-type and ASK1−/− mice, with or without angiotensin II infusion. The upper panels in A and B indicate representative TUNEL and Western blot in each group. Arrows in A indicate vascular endothelial apoptosis. Each bar represents mean ± SEM (n = 8 per group). *P < 0.05 vs Wild AII (−).

Table 1. Summary of the Effect of Angiotensin II Infusion on Survival Rate and Vascular Endothelial Injury in DS Rats

<table>
<thead>
<tr>
<th>Condition</th>
<th>Survival Rate (%)</th>
<th>Caspase-3 Positive Cells (%)</th>
<th>Diameter/Monomer (folds)</th>
<th>eNOS Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild AII (−)</td>
<td>100</td>
<td>0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>ASK1−/− AII (−)</td>
<td>75 ± 5</td>
<td>3</td>
<td>1.5 ± 0.5</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>Wild AII (+)</td>
<td>55 ± 5</td>
<td>5</td>
<td>1.5 ± 0.5</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>ASK1−/− AII (+)</td>
<td>25 ± 5</td>
<td>7</td>
<td>2.0 ± 0.5</td>
<td>50 ± 10</td>
</tr>
</tbody>
</table>

Abreviations are the same as in Figure 3.
sive heart failure, we initiated treatment with BH4, a specific NADPH oxidase inhibitor, in 20-week-old DS rats with overt heart failure (Figures 5 and 6 and supplemental Figures VIII through X). Of note are the observations that the suppression of vascular eNOS uncoupling by BH4 treatment at advanced stage of heart failure significantly improved cardiac diastolic dysfunction and prolonged survival rate of DS rats. Furthermore, BH4 treatment in DS rats with overt heart failure significantly reversed vascular endothelial dysfunction, and these beneficial effects were associated with the significant reduction of vascular superoxide and the restoration of eNOS activity. On the other hand, the significant NADPH oxidase inhibition by apocynin treatment or the vasodilation by hydralazine treatment did not significantly improve survival rate of DS rats, despite their similar blood pressure lowering effects to BH4. Differing from BH4 treatment, apocynin significantly inhibited NADPH oxidase activity, but did not improve vascular endothelial function, not prevent the progression of coronary remodeling, not diminish vascular superoxide, and not restore eNOS activity. These results provided the solid evidence that eNOS uncoupling, via the production of superoxide, is involved in the exacerbation of vascular endothelial injury in DS rats, and suggested that eNOS uncoupling may play some role in the pathophysiology of diastolic heart failure in DS rats.

NADPH oxidase is reported to be involved in angiotensin II–induced ROS generation, as reviewed. However, differing from our present study, previous studies have not examined animals at the stage of advanced vascular remodeling or heart failure. Therefore, the difference in the source of ROS between our present finding and previous findings might be explained by the difference in the stage of progression of vascular remodeling or cardiac dysfunction. Another possible reason is that our present findings might be specific for salt-sensitive hypertension or heart failure. Thus the relative role of eNOS uncoupling and NADPH oxidase in the generation of ROS seems to depend on the stage of vascular remodeling or the type of cardiovascular diseases.

**Study Limitation**

BH4 treatment of DS rats with diastolic heart failure improved cardiac diastolic dysfunction and survival rate, suggesting that vascular endothelial dysfunction caused by eNOS uncoupling may participate in the exacerbation of diastolic heart failure. However, the present study did not allow us to elucidate the potential role of vascular endothelial dysfunction in the pathogenesis of diastolic heart failure, because the main purpose of our present work was to examine the molecular mechanism of vascular endothelial injury in hypertensive diastolic heart failure. Hence, further study is needed to elucidate the accurate role of vascular endothelial injury in the pathophysiology of diastolic heart failure. Moreover, it remains to be determined whether or not our present findings are specific for diastolic heart failure.

In conclusion, in our current work, we obtained the evidence that AII-induced endothelial apoptosis and eNOS uncoupling are mediated by ASK1 activation and play a key role in the exacerbation of vascular injury in diastolic heart failure of salt-sensitive hypertension. Thus, our present work provided novel molecular mechanism underlying AII-induced vascular injury. Furthermore, ASK1 appears to be potentially the useful target for treatment of hypertensive diastolic heart failure.

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Disclosures
None.

References
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Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Dahl salt-sensitive hypertensive rats (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.

Male ASK1-/- mice and wild type mice (C57BL/6J) were used in the present study. Generation of ASK1-/- mice has been described previously. ASK1-/- mice were backcrossed into the C57BL/6J background at least 10 generations to reduce genetic variation.

Comparative effect of valsartan and amlodipine on vascular injury of DS rats fed high-salt diet

To elucidate the direct role of angiotensin II (AII) in vascular diseases of salt-sensitive hypertension, we compared the effect of valsartan (Novartis) and amlodipine (Pfizer) on DS rats. Twelve-week-old DS rats, which had fed a high-salt diet from 7 weeks of age, were given vehicle (0.5 % carboxymethyl cellulose [CMC]), valsartan (10 mg/kg/day), or amlodipine (1 mg/kg/day), by gastric gavage once a day for 4 weeks (until 16 weeks of age). Valsartan and amlodipine were suspended in 0.5 % CMC. In preliminary experiments, we found that valsartan and amlodipine at the above mentioned dose exerted comparable hypotensive effects in DS rats. Blood pressure and heart rate of the conscious rats were measured with the tail-cuff method (BP98A; Softron, Tokyo) at 3 to 5 hours after oral dosing, every week. After 4 weeks of drug treatment, DS rats were anesthetized with ether, arterial blood was immediately collected by cardiac puncture, and plasma was collected by centrifugation and stored at -80°C until measurement of plasma NO\textsubscript{2}/NO\textsubscript{3} (NO\textsubscript{x}). Then the carotid artery and the thoracic aorta were immediately excised to compare the effect of each treatment on vascular endothelial function, apoptosis, NADPH oxidase activity, superoxide, eNOS activity, eNOS dimer and monomer, and phosphorylation of ASK1. The heart was also removed to compare the effect of each drug on coronary arterial thickening.

Comparative effect of valsartan and amlodipine on survival rate of DS rats fed high-salt diet

Twelve-week-old DS rats, fed a high-salt diet from 7 weeks of age, were orally given vehicle, valsartan (10 mg/kg/day), or amlodipine (1 mg/kg/day) in the same manner as the above experiment, and survival rate was examined until 24 weeks of age. Animals were carefully monitored, and deaths were recorded every day. Survival rates of DS rats were compared among each group.
Effect of angiotensin II infusion on wild type and ASK1-/- mice

To examine the role of ASK1 in vascular oxidative stress, vascular endothelial function, apoptosis, and eNOS activity and uncoupling, we compared the effects of chronic angiotensin II (AII) infusion on wild type and ASK1-/- mice. AII (600 ng/kg/min) dissolved in saline or saline alone (as control) was subcutaneously infused to mice via osmotic minipump (ALZACO) for 4 weeks. Blood pressure and heart rate were periodically monitored throughout continuous AII infusion, with the tail-cuff method. After 4 weeks of AII infusion, mice were anesthetized with ether, and carotid artery and thoracic aorta were excised to examine vascular endothelial function, apoptosis, ASK1 phosphorylation, NADPH oxidase activity, p22phox expression, superoxide, eNOS uncoupling, eNOS activity, phospho-eNOS, and total eNOS.

Role of eNOS uncoupling in end-stage heart failure of DS rats

Previously, we and others 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. Therefore, using 20-week-old DS rats with overt heart failure, we examined the role of eNOS uncoupling in end-stage heart failure of DS rats. Tetrahydrobiopterin (BH4; sapropterin hydrochloride, Daiichi Suntory Pharma Co Ltd, Tokyo) (10 mg/kg/day), apocynin (0.3 mmol/kg/day), or hydralazine (20 mg/kg/day) in the drinking water was given to 20-week-old DS rats with overt heart failure, for 4 weeks (until 24 weeks of age). Blood pressure was measured with the tail-cuff method every week. During 4 weeks of drug treatment, animals were carefully monitored and the number of dead rats was recorded every day to examine survival rate of DS rats. After 4 weeks of drug treatment, surviving 24-week-old DS rats in each group were anesthetized with ether, and the carotid artery and the thoracic aorta were excised to compare vascular endothelial function, eNOS uncoupling, eNOS activity, superoxide, and NADPH oxidase activity. Furthermore, as a control, we also examined the effect of tetrahydroneopterin (H4N; Schircks Laboratories) (10 mg/kg/day), which has similar antioxidant properties to BH4 but is not directly linked to NOS coupling and activity, in DS rats.

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. 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 LV anterior and posterior walls (AW and PW, respectively) at the papillary muscle level to measure LV end-diastolic dimension, LV end-systolic dimension, fractional shortening, LV ejection fraction, LV 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 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.

**Vessel Ring Preparation and Organ Chamber Experiments**

Isometric tension studies were performed, as previously described. In brief, carotid arteries from DS rats or thoracic aortas from mice were cut into 5 mm rings with special care to preserve the endothelium, and mounted in organ baths filled with, modified Tyrode buffer (pH 7.4; NaCl 121 mmol/L, KCl 5.9 mmol/L, CaCl₂ 2.5 mmol/L, MgCl₂ 1.2 mmol/L, NaH₂PO₄ 1.2 mmol/L, NaHCO₃ 15.5 mmol/L, and D(+)-glucose 11.5 mmol/L) aerated with 95 % O₂ and 5 % CO₂ at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. A resting tension of 1 g was maintained throughout the experiment. Vessel rings were precontracted with L-phenylephrine (10⁻⁷ mol/L). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach) (10⁻⁹ mol/L to 10⁻⁴ mol/L) or sodium nitroprusside (10⁻⁹ mol/L to 10⁻⁴ mol/L) to obtain cumulative concentration-response curves.

**Vascular NADPH oxidase activity**

Aortic tissue was 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. Protein concentrations were measured by the method of Bradford.

**Measurement of in situ superoxide production, using the fluorescent dye dihydroethidium**

Carotid arteries, removed from DS rats or mice, were immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate carotid arterial superoxide levels in situ, as described in detail. Although this method is very popular and is frequently used for the determination of superoxide, this method has some limitations. To demonstrate the validity of our method, some sections were preincubated for 30 minutes with either PBS, 250 U/mL polyethylene-glycol superoxide dismutase (PEG-SOD) (Sigma-Aldrich), the cell-permeable superoxide scavenger, 5 mM Tiron (Dojindo), the cell-permeable superoxide scavenger, or 100 µmol/L No-nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich), before incubation with DHE. DHE fluorescence of arterial section was quantified using Lumina Vision version 2.2, analysis software. The mean fluorescence was quantified and expressed relative to values obtained from control rats.

**Measurement of ex vivo vascular superoxide production, using lucigenin-chemiluminescence**

Superoxide production in aortic segments was also measured, according to the lucigenin-enhanced chemiluminescence method. Segments of the thoracic aorta (20 mm) from DS rats were placed in modified Krebs/HEPES buffer (pH 7.4), and allowed to
equilibrate for 30 minutes at 37°C. After equilibration, the ring was placed in 1 mL of Krebs-HEPES buffer containing lucigenin (Sigma Chemical Co; 50 µmol/L) and equilibrated in the dark for 5 minutes at 37°C. The chemiluminescence was then recorded every 15 seconds for 5 minutes, with the use of a luminescence reader (BLR-201, Aloka). Lucigenin counts were expressed as cpm/mg dry wt vessel. To address the influence of eNOS-mediated superoxide production on vascular lucigenin-enhanced chemiluminescence, vessels were preincubated with L-NAME (10 µmol/L) for 30 minutes, as reported.

Preparation of arterial protein extracts and Western blot analysis of phospho-ASK1, phospho-eNOS, total eNOS, and p22phox

Our detailed method has been described previously. Briefly, after aortic protein extracts were subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows: anti-phospho ASK1, anti-phospho-eNOS (Ser-1177) (x 5000, BD Transduction Laboratories), anti-eNOS (x 5000, BD Transduction Laboratories), anti-α-tubulin (x 5000, CALBIOCHEM), anti-p22phox (x2500, SANTA CRUZ BIOTECHNOLOGY, INC). In individual samples, each value was corrected for that of α-tubulin.

Determination of eNOS dimer and monomer

eNOS dimer and monomer were separated, using low-temperature SDS-PAGE followed by Western blot analysis, as described previously.

For immunoblot analysis of the dimeric and monomeric form of eNOS protein, arterial samples were not heated and the temperature of the gel was maintained at 4 °C during electrophoresis (low-temperature SDS-PAGE). The proteins were transferred by semidry electroblotting to polyvinylidene difluoride membranes for 90 min. The blots were then blocked and incubated with anti-eNOS monoclonal antibody for overnight at 4 °C. Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (x5000, Santa Cruz Biotechnology). The antibody was visualized using an enhanced chemiluminescence method (ECL; Amersham Biosciences). The intensity of the bands was quantified using NIH Image analysis software v1.61.

NOS activity (arginine-to-citrulline conversion) and plasma NOx

Ca²⁺-dependent or -independent NOS activity was determined by measuring the conversion of [3H]-arginine to [3H]-citrulline, using a NOS assay kit (Calbiochem), as described.

To determine plasma NO, plasma NOx was measured by a commercially available kit (Dojindo, Co).

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 coronary arterial thickness, as previously described.

Apoptosis in carotid artery of DS rats was detected with the TdT-mediated dUTP nick-end labeling (TUNEL) by utilizing in situ Apoptosis Detection Kit (Takara, Shiga Japan). To detect vascular apoptosis of mice, frozen aortic sections from wild type and ASK1−/− mice were incubated with 1:100 dilution of rabbit anti-active caspase-3 antibody (R&D) overnight at 4°C. After washing, FITC-conjugated secondary antibody (DAKO, Glostrop, Denmark) were applied for 60 minutes in the dark, and sections were washed and coverslipped. Fluorescence images were acquired using fixed exposure times using a CCD camera attached to a Zeiss Axiovert inverted microscope. To quantify vascular apoptosis, at least 4 cross sections of each vessel immunohistochemically stained with TUNEL, or with an antibody against active caspase-3 were examined, and positive cells per section were counted. The average number of apoptotic cells in the intima per section was calculated.

**Measurement of bipterin content in vascular tissue**

Aortic bipterin levels were measured by high-performance liquid chromatography (HPLC) with fluorescent detection after iodine oxidation in acidic or alkaline conditions, as described previously. Tissue was homogenized in cold extract buffer (50mmol/L Tris-HCl of pH 7.4, 1mmol/L EDTA, ascorbic acid), and centrifuged at 16000g for 15 minutes at 4°C. The amount of BH4 was determined from the difference between total (BH4 plus BH2 plus bipterin) and alkaline-stable oxidized (BH2 plus bipterin) bipterin.

**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.). In all other data, 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 Figure Legends

**Online Fig. I** Effect of valsartan and amlodipine on blood pressure (A), vascular endothelium-dependent relaxation (B), coronary arterial thickening (C), and survival rate (D) of DS rats fed a high-salt diet.

High Na indicates DS rats fed an 8% NaCl diet from 7 weeks of age. Low Na indicates DS rats fed a 0.3% NaCl diet throughout the experiments. Val, Am, and Veh indicate DS rats treated with valsartan, amlodipine, and vehicle, respectively, from 12 to 16 weeks of age. In (A), (B), and (C), values are mean±SEM (n=6-8). (D) DS rats were orally given vehicle, (Veh), valsartan (Val), or amlodipine (Am) for 12 weeks (from 12 to 24 weeks of age), and survival rate of DS rats were compared among each group. Each group included 13 animals.

**Online Fig. II** Effect of valsartan (Val) and amlodipine (Am) on vascular NADPH oxidase activity (A) and vascular superoxide (B) of DS rats.

The upper panels in (B) indicate representative fluorescence photomicrographs in each group. Each bar represents mean±SEM (n= 6-8). Abbreviations are the same as in Online Fig.I. NS, not significant.

**Online Fig. III** Substantial reduction of dihydroethidium fluorescence in vascular sections by pretreatment with PEG-SOD and Tiron

Pretreatment with PEG-SOD or Tiron substantially reduced dihydroethidium fluorescence in vascular sections. The intensity of dihydroethidium fluorescence in vascular sections from salt-loaded DS rats was reduced to similar levels to that from control DS rats, by treatment with PEG-SOD or Tiron. These data confirmed that the increase in vascular dihydroethidium fluorescence of salt-loaded DS rats was attributed to the increase in superoxide production. L-NAME treatment significantly attenuated vascular dihydroethidium fluorescence in salt-loaded DS rats, but not in control DS rats. Each bar represents mean±SEM (n= 6-8).

**Online Fig. IV** L-NAME markedly reduced lucigenin chemiluminescence in vascular segments from salt-loaded DS rats

Lucigenin-enhanced chemiluminescence indicated that vascular superoxide production was larger in salt-loaded DS rats than control DS rats, being in good agreement with the findings obtained by the method with the fluorescent probe dihydroethidium. Furthermore, vascular superoxide production in salt-loaded DS rats was markedly reduced by treatment with L-NAME, indicating that the increase in vascular superoxide production in salt-loaded DS rats was at least in part mediated by eNOS uncoupling. Each bar represents mean±SEM (n= 6-8).

**Online Fig. V** Effect of angiotensin II infusion on blood pressure (A) and vascular ASK1 phosphorylation (B) of wild and ASK1-/- mice.

The upper panels in (B) indicate representative Western blot in each group, and each band
was obtained from the same gel. Wild (-), wild type mice without angiotensin II infusion; Wild (+), wild type mice infused with angiotensin II; ASK1-/- (-), ASK1-/- mice without angiotensin II infusion; ASK1-/- (+), ASK1-/- mice infused with angiotensin II. Values are mean±SEM (n= 8 in each group). AII, angiotensin II infusion. N.D., not detected.

**Online Fig. VI** Effect of angiotensin II infusion on vascular NADPH oxidase activity (A), vascular p22phox (B), and vascular superoxide (C) of wild type and ASK1-/- mice, with or without angiotensin II infusion.

The upper panels in (B) indicate representative Western blot, which was obtained from the same gel. The upper panels in (C) indicate fluorescence photomicrographs in each group. Abbreviations are the same as in Online Fig.V. Each bar represents mean±SEM (n=8 in each group).

**Online Fig. VII** Effect of angiotensin II infusion on vascular phospho-eNOS (p-eNOS) (A) and total eNOS (B) of wild type and ASK1-/- mice, with or without angiotensin II infusion.

Abbreviations are the same as in Online Fig.V. The upper panels in (A) and (B) indicate representative Western blot in each group, and each band in (A) and (B) was obtained from the same gel. Each bar represents mean±SEM (n=8 per group).

**Online Fig. VIII** Effect of tetrahydrobiopterin, apocynin, and hydralazine on blood pressure of DS rats with overt heart failure.

Twenty-week-old DS rats with overt heart failure were given vehicle (n=14), tetrahydrobiopterin (n=12), apocynin (n=14), or hydralazine (n=15) for 4 weeks (until 24 weeks of age). Veh, vehicle treatment; BH4, tetrahydrobiopterin treatment; Apo, apocynin treatment; Hyd, hydralazine treatment. Each value represents mean±SEM.

**Online Fig. IX** Effect of tetrahydrobiopterin, apocynin, and hydralazine on cardiac function of DS rats with overt heart failure, estimated by echocardiography.

Twenty-week-old DS rats with overt heart failure were given vehicle (n=14), tetrahydrobiopterin (n=12), apocynin (n=14), or hydralazine (n=15) for 2 weeks, and E/A (A), LVDd (B), and FS (C) were estimated by echocardiography. Abbreviations are the same as in Online Fig.VIII. Each value represents mean±SEM (n=5 in Veh and Hyd, n=6 in Apo, n=10 in BH4).

**Online Fig. X** Effect of tetrahydrobiopterin, apocynin, and hydralazine on acetylcholine-induced vascular endothelium-dependent relaxation (A) and coronary arterial thickening (B) of DS rats with overt heart failure.

Twenty-week-old DS rats with overt heart failure were given vehicle (n=14), tetrahydrobiopterin (n=12), apocynin (n=14), or hydralazine (n=15) for 4 weeks (until 24 weeks of age). After 4 weeks of each drug treatment, we examined vascular endothelial function (A) and coronary arterial thickening (B) of surviving DS rats in each group (tetrahydrobiopterin [n=7], apocynin [n=5], hydralazine [n=6]). Veh, vehicle treatment; BH4, tetrahydrobiopterin treatment; Apo, apocynin treatment; Hyd, hydralazine treatment.
Low Na indicates DS rats fed a 0.3% NaCl diet, throughout the experiments. Pre indicates drug-untreated 20-week-old DS rats with heart failure. Each value represents mean±SEM.
<table>
<thead>
<tr>
<th></th>
<th>Total biopterin (pmol/mg protein)</th>
<th>BH4 (pmol/mg protein)</th>
<th>Oxidized biopterin (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Na (n=5)</td>
<td>4.84±0.37</td>
<td>3.58±0.27</td>
<td>1.26±0.37</td>
</tr>
<tr>
<td>High Na (n=5)</td>
<td>4.43±0.59</td>
<td>1.64±0.19*</td>
<td>2.79±0.53*</td>
</tr>
</tbody>
</table>

Low Na, 16-week-old DS rats fed a low-salt diet; High Na, 16-week-old DS rats fed a high-salt diet from 7 weeks of age. * p < 0.01, + p < 0.05 vs Low Na. Values are mean±SEM.
Online Figure I

(A) (mmHg)

(B) (%)

(C)

(D) (%)
(A) NADPH oxidase activity (CPM/mg) in Low and High Na conditions. 

- Veh: Vehicle
- Val: Valinol
- Am: Amiloride

† P<0.05, * P<0.01 vs Veh

(B) Relative fluorescence (%)

- Low Na
- High Na

† P<0.05

* P<0.01 vs Veh
Online Figure III

The figure shows the relative fluorescence intensity (%). The bars represent different treatments: PBS, SOD, Tiron, and L-NAME. The x-axis indicates Low Na and High Na conditions. The y-axis shows the relative fluorescence intensity. Significant differences are indicated by P<0.01.
Low Na  High Na
(-)  L-NAME  (+)  (-)  (+)  
P<0.01  P<0.01

Online Figure IV
(A) Blood pressure

Wild (-)  Wild (+)  ASK1-/- (-)  ASK1-/- (+)

Weeks

0 1 2 3 4

Blood pressure (mmHg)

* P<0.01 vs Wild (-)
† P<0.01 vs ASK1 -/- (-)

(B) P-ASK1

α-tubulin

* P<0.01 vs Wild (-)
Online Figure VI

† P<0.05, *P<0.01 vs Wild, AII (-) □ P<0.05, ‡ P<0.01 vs ASK1-/-, AII (-) NS, not significant

(A) NADPH oxidase (CPM/mg)

(B) p22phox (AU)

(C) Relative fluorescence (%)
Online Figure VIII

Blood pressure (mmHg)

* P < 0.01 vs Veh

Weeks

0 1 2 3 4

Veh
Hyd
Apo
BH4
(A) Low Na BH4 Apo Relaxation 0 20 40 60 80 100 (%)
Acetylcholine (-log mol/L)

(B) Arterial thickening Low Na Hyd Pre High Na Apo BH4

† P<0.05 * P<0.01

Online Figure X