Abstract—Reactive oxygen species have an important pathogenic role in organ damage. We investigated the role of oxidative stress via nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase in the kidney of the Dahl salt-sensitive (DS) rats with heart failure (DSHF). Eleven-week-old DS rats fed an 8%-NaCl diet received either vehicle or imidapril (1 mg/kg per day) for 7 weeks. The renal expression of the NAD(P)H oxidase p47phox and endothelial NO synthase were evaluated. In DSHF rats, associated with increased renal angiotensin II, mRNA and protein expression of NAD(P)H oxidase p47phox were enhanced with an increase in renal lipid peroxidation production (0.33±0.03 versus 0.22±0.01 nmol/mg protein, P<0.05) and urinary excretion of hydrogen peroxide (26.9±6.6 versus 9.5±2.1 U/mg creatinine, P<0.01) compared with levels in Dahl salt-resistant rats. The endothelial NO synthase expression was decreased in the kidney. Treatment with imidapril reduced renal angiotensin II and NAD(P)H oxidase expression and the oxidative products (kidney lipid peroxidation product: 0.16±0.02, P<0.001; urinary hydrogen peroxide: 3.1±0.2, P<0.01 versus DSHF rats). Imidapril significantly decreased albuminuria and reduced glomerulosclerosis without changes in the blood pressure. In conclusion, DSHF rats showed increased oxidative stress in the kidney via NAD(P)H oxidase. Blockade of local angiotensin II with subpressor dose of imidapril inhibited NAD(P)H oxidase and prevented renal damage. (Hypertension. 2002;40:834-839.)

Key Words: heart failure ■ nitric oxide synthase ■ angiotensin-converting enzyme ■ rats, Dahl ■ angiotensin II Oxidative Stress in Dahl Salt-Sensitive Rat With Heart Failure

Akihiro Tojo, Maristela Lika Onozato, Naohiko Kobayashi, Atsuo Goto, Hiroaki Matsuoka, Toshiro Fujita

Congestive heart failure causes proteinuria and renal dysfunction via kidney hypoperfusion. However, there are few studies that investigated the mechanisms of renal damage in congestive heart failure. Recently, an animal model of congestive heart failure has been established in Dahl salt-sensitive (DS) rats fed a high-salt diet. In this model, left ventricular (LV) hypertrophy is observed by echocardiography at 11 weeks after induction, and LV dilatation with decreased cardiac output at 18 weeks. In the heart, ACE and endothelin-1 mRNA expression was increased significantly in DS rats with heart failure (DSHF), and ACE inhibitor (ACEI) ameliorated the development of heart failure, reducing ACE and endothelin-1 mRNA expression. Furthermore, in the DSHF rat, NO production by endothelial NO synthase (eNOS) was decreased in the heart, enhancing type IV collagen production and inducing LV fibrosis and remodeling. Thus, enhanced local angiotensin (Ang) II and reduction of NO may have an important role in the development of congestive heart failure in DSHF rats.

Recently, oxidative stress has been explored in the mechanism of heart failure. The sources of oxidative stress include vascular nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase, xanthine oxidase, auto-oxidation of catecholamines, NOS activation, or mitochondrial leakage. In the kidney, NAD(P)H oxidase exists not only in the migrating macrophages but also in the renal vessels, glomerular podocytes, mesangial cells, and distal tubules, and it produces superoxide anion. The radical production via NAD(P)H oxidase has an important role in the renal damage of diabetic rats, and ACEI suppressed renal NAD(P)H oxidase and reduced microalbuminuria. Similarly, we hypothesized that in the kidney of congestive heart failure model of DS rats fed a high-salt diet, local Ang II level might be elevated via renal ischemia and might enhance NAD(P)H oxidase expression and cause renal damage. In the present study, we investigated the expression of NAD(P)H oxidase and NOS isoforms in the kidney of the DSHF rats and evaluated the effect of the ACEI imidapril on the renal damage.
NaCl until the age of 6 weeks. Kidneys from 4 prehypertensive rats were removed at 6 weeks to investigate radical production. Thereafter, they were fed a diet containing 8% NaCl until the age of 18 weeks. At age 11 weeks, when LV hypertrophy develops, DS rats were randomly divided into two groups: rats treated with DSHF (n = 10) and rats treated with imidapril (1 mg/kg per day, Tanabe Pharmaceutical Co; DSHF + ACEI; n = 10) in drinking water. They were treated until the age of 18 weeks, when LV failure was established.4,5,9 Age-matched Dahl salt-resistant rats fed the same diet served as controls (n = 10).

**Hemodynamic Measurements and Urine Collection**

The systolic blood pressure was measured by tail-cuff method; 24-hour urinary collection and transthoracic echocardiography evaluating the LV end-diastolic diameter and fractional shortening were performed at 18 weeks, as described previously.6,7,9

**Reverse Transcription–Polymerase Chain Reaction for NAD(P)H Oxidase p47phox in the Kidney**

At 18 weeks after physiological data sampling, 5 rats were anesthetized with sodium pentobarbital (50 mg/kg body weight IP), and the kidneys were immediately excised and frozen in liquid nitrogen. Total RNA was prepared, and reverse transcription–polymerase chain reaction (RT-PCR) was performed by standard methods, as described previously8,9 by use of a synthetic gene-specific primer for NAD(P)H oxidase p47phox: upstream primer, 5′-GGCAGGACCTGTCGGAGAAGGTGG-3′/H11032 and downstream primer, 5′-TGAAGGATGATGGGGCCTGTGATG-3′/H11032. Parallel amplification of rat GAPDH was performed for normalization. The quantitative analysis of p47phox protein in the kidney was enhanced in the kidney of DSHF rats. Imidapril treatment significantly suppressed Ang II level in the kidney. The renal tissue Ang II level was markedly higher in the kidney of DSHF rats than in controls, and treatment with imidapril significantly decreased Ang II level by Western blot and Northern blot analyses.

**Statistics**

All data were expressed as mean±SE. The mean values were compared among the 3 groups using ANOVA followed by the Bonferroni post-hoc test. Probability values <0.05 were required for statistical significance.

**Results**

**Physiological Data of DSHF Rats and Effects of Imidapril**

As shown in the Table, DSHF rats at 18 weeks demonstrated severe hypertension, a significant increase in LV weight and end-diastolic diameter, and a significant reduction of fractional shortening, demonstrating established congestive heart failure. The subpressor dose of imidapril did not decrease systolic blood pressure. However, LV weight, end-diastolic diameter, and fractional shortening were significantly alleviated. The renal hemodynamics, as shown by creatinine clearance, were slightly decreased in DSHF rats, and treatment with imidapril did not ameliorate creatinine clearance. The tissue Ang II level was markedly higher in the kidney of DSHF rats than in controls, and treatment with imidapril significantly suppressed Ang II level in the kidney. The renal Ang II level in the 6-week-old prehypertensive DS rats fed a low-salt diet was not increased (1175±37 pg/g kidney weight, n=4; P = NS versus control).

**NAD(P)H Oxidase p47phox and NOS Expression in DSHF Rats**

NAD(P)H oxidase component p47phox was weakly expressed in the glomerular cells and distal tubules in the kidney of control rats, whereas its expression was markedly enhanced in the kidney of DSHF rats. Imidapril treatment reduced NAD(P)H oxidase expression in glomeruli (Figure 1). The quantitative analysis of p47phox protein in the kidney by Western blot showed a specific band corresponding to a molecular weight of 47 kDa. As shown in Figure 2, densitometry of the band confirmed an increase in the protein amount in DSHF rats compared with control rats (0.368±0.012 versus 0.313±0.011 arbitrary units; P<0.005). This was suppressed significantly by imidapril (0.323±0.005; P<0.01 versus DSHF).
The enhanced NAD(P)H oxidase p47phox mRNA in the kidney of DSHF rats was also confirmed by RT-PCR. Imidapril treatment reduced mRNA expression of NAD(P)H oxidase p47phox in kidney of DSHF rats (Figure 3). The eNOS immunoreactivity was significantly decreased in endothelial cells of renal artery of DSHF rats compared with control rats (Figure 4). Imidapril treatment enhanced eNOS immunoreactivity to control levels (Figure 4). The NO production evaluated by nitrite production in the kidney was decreased in DSHF rats, and it was reversed to the control level by ACEI treatment (Table). On the other hand, the NO production evaluated by nitrite production in the kidney was decreased in DSHF rats, and it was reversed to the control level by ACEI treatment (Table). The enhanced NAD(P)H oxidase expression in the kidney has a functional activity as the source of radical production. As a result of enhanced NAD(P)H oxidase expression in the kidney, lipid peroxidation (LPO) production in the kidney tissue was significantly increased in DSHF rats (Table). This was reduced significantly by treatment with imidapril to levels not different from those of control rats. The urinary excretion of LPO and hydrogen peroxide was significantly increased in DSHF rats, and the imidapril treatment significantly reduced hydrogen peroxide production (Table). The prehypertensive DS rat showed less renal LPO production, with a faint NAD(P)H oxidase expression (0.07±0.01 nmol/mg protein; P<0.001 versus control). The prehypertensive DS rat showed less renal LPO production, with a faint NAD(P)H oxidase expression (0.07±0.01 nmol/mg protein; P<0.001 versus control).

**Oxygen Radical Production and Renal Damage**

As shown in Figure 6, NAD(P)H oxidase in the glomerulus produces hydrogen peroxide evaluated by DCF generation, and apocynin (an inhibitor of NAD(P)H oxidase) inhibited hydrogen peroxide production. This indicated that the expression of NAD(P)H oxidase in the kidney has a functional activity as the source of radical production. As a result of enhanced NAD(P)H oxidase expression in the kidney, lipid peroxidation (LPO) production in the kidney tissue was significantly increased in DSHF rats (Table). This was reduced significantly by treatment with imidapril to levels not different from those of control rats. The urinary excretion of LPO and hydrogen peroxide was significantly increased in DSHF rats, and the imidapril treatment significantly reduced hydrogen peroxide production (Table). The prehypertensive DS rat showed less renal LPO production, with a faint NAD(P)H oxidase expression (0.07±0.01 nmol/mg protein; P<0.001 versus control).

**Light Microscopy Morphological Changes and Urinary Protein Excretion**

As shown in Figure 7, DSHF rats showed marked mesangial matrix increment, glomerulosclerosis, arteriolosclerosis, and tubulointerstitial damage, with many hyaline casts in the tubules. Treatment with imidapril obviously ameliorated these changes.

Morphological changes were accompanied by changes in urinary albumin excretion. DSHF rats presented markedly increase in urinary albumin excretion, which was significantly reduced with treatment with imidapril (Table).

### Physiological Data

<table>
<thead>
<tr>
<th></th>
<th>Control (n=5)</th>
<th>DSHF (n=5)</th>
<th>DSHF + ACEI (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>427±7</td>
<td>294±4*</td>
<td>386±5†</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>127±2</td>
<td>251±6*</td>
<td>247±7*</td>
</tr>
<tr>
<td>LV weight, g</td>
<td>0.87±0.02</td>
<td>1.35±0.02*</td>
<td>1.08±0.03‡</td>
</tr>
<tr>
<td>End-diastolic diameter, mm</td>
<td>7.4±0.07</td>
<td>9.44±0.38*</td>
<td>7.59±0.26§</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>42.3±0.8</td>
<td>25.2±0.7*</td>
<td>38.5±1.4§</td>
</tr>
<tr>
<td>Cr clearance, mL/min</td>
<td>3.60±0.91</td>
<td>2.35±0.64</td>
<td>1.45±0.18§</td>
</tr>
<tr>
<td>Renal ang II, pg/g kidney weight</td>
<td>1302±186</td>
<td>2442±126§</td>
<td>1350±462‡</td>
</tr>
<tr>
<td>NOx in the kidney, µmol/mg protein</td>
<td>1.63±0.15</td>
<td>0.52±0.08#</td>
<td>1.41±0.22†</td>
</tr>
<tr>
<td>Renal LPO production, nmol/mg protein</td>
<td>0.22±0.01</td>
<td>0.33±0.03§</td>
<td>0.16±0.02‡</td>
</tr>
<tr>
<td>Urinary LPO product, nmol/mg Cr</td>
<td>2.0±0.3</td>
<td>3.0±0.4§</td>
<td>1.4±0.1†</td>
</tr>
<tr>
<td>Urinary H2O2, Fl unit/mg Cr</td>
<td>9.5±2.1</td>
<td>26.9±6.6#</td>
<td>3.1±0.2†</td>
</tr>
<tr>
<td>Urinary albumin excretion, mg/mg Cr</td>
<td>0.6±0.2</td>
<td>7.7±1.7*</td>
<td>2.4±0.3§</td>
</tr>
</tbody>
</table>

Values are mean±SE. Cr indicates creatinine; LV, left ventricle; Fl, fluorescence intensity. §P<0.05, #P<0.01, *P<0.001 vs control; †P<0.05, ‡P<0.01, ††P<0.001 vs DSHF rats.

![Figure 1. Immunohistochemistry for NAD(P)H oxidase cytosolic component p47phox in the kidney: control (a), DSHF rat fed an 8% NaCl diet (b), and DSHF + ACEI rat (c). Bar, 50 μm; magnification, ×150.](image-url)
DSHF rats,5 which might have contributed to the increased ACE mRNA and protein levels were increased in the heart of may be caused by both heart failure and hypertension. Tissue with cardiac ultrasonography; therefore, the renal damage 0.001 /H11021 oxidase cytosolic component p47phox in the kidney. * P /H11021 RT-PCR analysis for mRNA expression for NAD(P)H oxidase expression and its oxidative products. This increased oxidative stress may cause renal damage, because subpressor dose of the ACEI imidapril prevented the renal damage with inhibition of NAD(P)H oxidase and its products. Proteinuria and reduction of renal function are often observed in the patients with severe heart failure. However, the mechanism of renal damage has not been elucidated. In the heart failure model of DS rats fed 8% sodium, LV hypertrophy is observed at 11 weeks, and LV dilatation and reduction of fractional shortening are observed at 18 weeks.1–5 We confirmed the establishment of heart failure with cardiac ultrasonography; therefore, the renal damage may be caused by both heart failure and hypertension. Tissue ACE mRNA and protein levels were increased in the heart of DSHF rats,5 which might have contributed to the increased tissue Ang II level. The decreased cardiac output lead to the activation of the plasma and the kidney renin-angiotensin system.13 We showed that the tissue Ang II level in the kidney was significantly higher in DSHF rats than in controls.

The increased local Ang II has an important role in the progression of renal damage via activation of tumor growth factor-β and other growth factors.14–17 Ang II can also cause renal damage via enhanced oxidative stress.8 NAD(P)H oxidase is the major source of oxidative stress and is stimulated by Ang II in the vascular smooth muscle cells.18 We have previously demonstrated that NAD(P)H oxidase expression is enhanced in the kidney of the hypertension model, spontaneously hypertensive rats.7 NAD(P)H oxidase cytosolic component p47phox binds to the membrane components, p22phox and gp91phox, and it is an important modulator of the activity of this enzyme.19,20 It is expressed mainly in podocytes, endothelium, distal convoluted tubules and fibroblast.7 Therefore, in the present study, we focused on p47phox and showed that its mRNA and protein expression were enhanced in the kidney of DSHF rats. In the vasculature, Ang II upregulates not only cytosolic components p47phox and p67phox21 but also membrane component 22phox.22 As a result of the enhanced expression of NAD(P)H oxidase, urinary LPO excretion and H2 O2 production in the kidney were significantly higher in DSHF rats than in controls. The kidney in early stage of diabetes also demonstrated enhanced NAD(P)H oxidase and its products, which was inhibited by ACEI or Ang II receptor blocker. Therefore, the enhanced oxidative stress is not a specific phenomenon in the kidney of heart failure or hypertension, but may depend on increased tissue Ang II level.

Actually, prehypertensive 6-week-old DS rats fed a low-salt diet did not show an increase in renal Ang II but were rather slightly lower compared with the control, ie, Dahl salt-resistant rats fed a high-salt diet. NAD(P)H oxidase p47phox expression was faint in prehypertensive DS rats, and LPO production in the kidney was less than that in controls. Thus, increased NAD(P)H oxidase expression and oxidative stress production in the kidney of DSHF rats may depend on both high-salt diet and the increased renal Ang II level associated with heart failure and hypertension.

NO produced by eNOS shows renoprotective effects in various kinds of nephritis and hypertension models.23–26 In the DSHF rats, we demonstrated that eNOS in the renal vasculature was significantly decreased. This is consistent with the results that eNOS expression was decreased in the heart of DSHF rats.4 The reduction of eNOS in the kidney is owing to hypertensive endothelial damage26 and high-salt diet;13 thus, it may not specific to heart failure. Surprisingly, nNOS in macula densa was enhanced in DSHF rats, even though nNOS expression has been reported to be decreased in DS rats compared with Dahl salt-resistant rats.27 and its function in the kidney was suppressed according to the development of hypertension.28,29 We have also demonstrated that nNOS in macula densa was suppressed in the kidney of rats fed a high-salt diet, and Ang II did not change its expression.11 The other models of hypertension, such as DOCA-salt hypertensive rats and the chronic NO blocking model, also showed a decrease in nNOS in macula densa,23,30

![Figure 2](image-url) Western blot analysis for NAD(P)H oxidase cytosolic component p47phox in the kidney homogenates. The average of density of band at molecular weight 47 kDa was calculated from 5 rats in each group. * P < 0.005 vs control; † P < 0.01 vs DSHF.

![Figure 3](image-url) RT-PCR analysis for mRNA expression for NAD(P)H oxidase cytosolic component p47phox in the kidney. * P < 0.001 vs control; † P < 0.001 vs DSHF.

**Discussion**

In the present study, we demonstrated the importance of oxidative stress in the renal damage associated with heart failure in DS rats. Tissue Ang II level was significantly increased in the kidney of heart failure rats and stimulated NAD(P)H oxidase expression and its oxidative products. This increased oxidative stress may cause renal damage, because subpressor dose of the ACEI imidapril prevented the renal damage with inhibition of NAD(P)H oxidase and its products.

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However, in our model of DSHF rats fed a high salt-diet, nNOS increased in macula densa. This increase in nNOS may be a specific phenomenon of heart failure in the DSHF rats. Further studies are necessary to elucidate the precise mechanism of enhancement of nNOS in DSHF rats.

The severe renal damage with glomerulosclerosis, arteriolosclerosis, and proteinuria in DSHF rats could be ascribed to the increased oxidative stress and to hypertension. The decreased eNOS in the kidney also caused reduction of renal NO production and reduction of NO renoprotective effect and may have enhanced renal damage. The subpressor dose of the ACEI imidapril significantly suppressed the glomerulosclerosis and proteinuria with a significant downregulation of NAD(P)H oxidase in the kidney, reducing urinary LPO production. The mechanism of ACEI to suppress oxidative stress in DSHF rats was owing to reduction of Ang II levels in the kidney, which stimulated NAD(P)H oxidase activity. The expression of eNOS and renal NO production were also enhanced by ACEI in the kidney of DSHF rats. These results demonstrated that renal damage in DSHF rats is partly explained by enhanced oxidative stress via NAD(P)H oxidase and reduction of the renoprotective effect of NO. Similarly, we have recently reported that both ACEI and Ang II receptor blocker inhibited oxidative stress in the kidney of diabetic nephropathy by reducing the expression of NAD(P)H oxidase and showed reduction of albuminuria. In the heart, tissue ACE expression was enhanced in the DSHF rat, and ACEI and angiotensin II receptor blocker (ARB) had a protective effective action on myocardial remodeling, perivascular fibrosis, and wall-to-lumen ratio of coronary arterioles via suppression of local Ang II. Thus, suppression of tissue Ang II has a beneficial effect not only in the kidney but also in the heart of DSHF rats. We provide an evidence that renoprotective effect of ACEI may partly depend on the suppression of oxidative stress by inhibition of NAD(P)H oxidase expression that is activated by Ang II.

**Perspectives**

In the present study, we provided one of the mechanisms of renoprotection of imidapril in the renal damage associated with hypertensive heart failure. The renal Ang II and its stimulation of NAD(P)H oxidase and oxidative stress production have an important pathogenical role in the renal damage of DSHF rats. Not only suppression of renal Ang II by ACEI
inhibitor or Ang II receptor blocker but also direct suppression of renal NAD(P)H oxidase expression is a promising strategy against renal damage associated with high renal Ang II levels as shown in hypertension, heart failure, and diabetes.

Acknowledgments

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References


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