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

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.

Methods

Animal Preparation
All experimental procedures were conducted in accordance with the Guide for Animal Experimentation (Faculty of Medicine, The University of Tokyo, Japan). Male inbred DS rats (Eisai Co Ltd, Tokyo, Japan; n = 24) were weaned and fed a diet containing 0.3%...
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 in 2 groups: rats treated with vehicle (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.3,5,9 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.4, 5, 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 previously6, 8 by use of a synthetic gene-specific primer for NAD(P)H oxidase p47phox: upstream primer, 5'-GGCACAGGACCTGTCGGAGAAGGTGG-3' and downstream primer, 5'-TGAAGGATGATGGGGCCTGTGATG-3' for the same RNA samples.

**Light Microscopy Morphological Study and Immunohistochemistry**

Other 5 rats from each group were anesthetized, and the kidneys were flushed with PBS. The right kidney was removed and frozen for Western blot, and the left kidney was perfused with periodate-lysine-paraformaldehyde solution and immersed in this solution overnight at 4°C. The tissues were embedded in paraffin for periodic acid–Schiff staining and light microscopic immunohistochemistry using a monoclonal antibody against neuronal NOS (nNOS), eNOS in a 1:1000 dilution, and the density of the bands was quantified by using densitometry. The intensity of the band of each gene was expressed relative to the corresponding densities of the GAPDH bands from the same RNA samples.

**Western Blot**

Western blotting was performed as we described previously.8, 10, 11 The kidney cortex was homogenized, and 25 μg of protein was applied to 4% to 20% gradient gel (Daichi Pure Chemicals Co) and electrobotted to nitrocellulose membranes. The membranes were stained with monoclonal antibody for NAD(P)H oxidase p47phox and eNOS in a 1:1000 dilution, and the density of the bands was analyzed by use of National Institutes of Health Image analyzer computer program.

**Measurement of Lipid Peroxidation, Hydrogen Peroxide, Nitrite, Creatinine, and Albuminuria**

All procedures for measurements were described in detail in our previous reports.8-12 Briefly, the lipid peroxidation products were measured by thiobarbituric acid method. After precipitation of proteins, 100 μL of urine or kidney homogenate was incubated with 100 μL of 4% sodium dodecyl sulfate, 400 μL of 20% acetic acid at pH 3.5, and 400 μL of 0.8% 2-thiobarbituric acid (Wako Pure Chemical Industries) for 60 minutes at 95°C. The malondialdehyde formation was measured by spectrophotometry (Hitachi H-2000) with an excitation/emission wavelength at 515/553 nm. The hydrogen peroxide production was measured by oxidation of nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). Samples were incubated with 16 μL/mL final concentration of DCFH-DA for 20 minutes at 37°C, and highly fluorescent DCF formed in the presence of hydrogen peroxide was measured in a spectrophotometer with wavelength at 485/535 nm.8 Nitrite production in the kidney homogenate was measured according to the Griess method. Protein was precipitated by adding an equal amount of 0.3 N NaOH and 5% ZnSO4; supernatant was reacted with Griess solution, and nitrite was measured by a spectrophotometer with wavelength at 540 nm.24 Creatinine in urine and blood was measured by the Jaffe method by using a spectrophotometer. Urinary albumin was quantified by enzyme-linked immunosorbent assay kit (Panaform Laboratory). The Ang II concentration in the kidney homogenate was measured by radioimmunoassay method (SRL), and the total amount of Ang II was corrected by kidney weight. To detect the activity of radical production by NAD(P)H oxidase, 5 μM frozen kidney section was incubated with NAD(P)H oxidase inhibitor (1 mmol/L apocynin) or PBS for 30 minutes and then incubated with DCFH-DA for 30 minutes. After washing with PBS, hydrogen oxide production was evaluated with DCF by a fluorescence microscope (Nikon, E600).

**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 ameliorated. 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 expression in renal vasculature. Western blot analysis (Figure 5) confirmed the decreased eNOS expression in DSHF rats compared with control rats (0.287 ± 0.003 versus 0.360 ± 0.012 arbitrary units; P < 0.005), which was restored to values not different from control rat levels 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 vs control) (Table).

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 increased in urinary albumin excretion, which was significantly reduced with treatment with imidapril (Table).

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.
DSHF rats, which might have contributed to the increased ACE mRNA and protein levels were increased in the heart of 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. We confirmed the establishment of heart failure in DS rats fed a high-salt diet; thus, it may not specific to heart failure. Surprisingly, we have also demonstrated the development of hypertension. We have previously demonstrated that NAD(P)H oxidase expression is enhanced in the kidney of the hypertension model, spontaneously hypertensive rats. We have previously demonstrated that NAD(P)H oxidase expression is enhanced in the kidney of the hypertension model, spontaneously hypertensive rats. We have also demonstrated enhanced oxidative stress with inhibition of NAD(P)H oxidase and its products. 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 Ang II did not change its level. 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. Ang II can also cause renal damage via enhanced oxidative stress. NAD(P)H oxidase is the major source of oxidative stress and is stimulated by Ang II in the vascular smooth muscle cells. We have previously demonstrated that NAD(P)H oxidase expression is enhanced in the kidney of the hypertension model, spontaneously hypertensive rats. It is expressed mainly in podocytes, endothelium, distal convoluted tubules and fibroblast. 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 p67phox but also membrane component 22phox. As a result of the enhanced expression of NAD(P)H oxidase, urinary LPO excretion and H2O2 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.

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. 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. 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, 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. 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. Ang II can also cause renal damage via enhanced oxidative stress. NAD(P)H oxidase is the major source of oxidative stress and is stimulated by Ang II in the vascular smooth muscle cells. We have previously demonstrated that NAD(P)H oxidase expression is enhanced in the kidney of the hypertension model, spontaneously hypertensive rats. It is expressed mainly in podocytes, endothelium, distal convoluted tubules and fibroblast. 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 p67phox but also membrane component 22phox. As a result of the enhanced expression of NAD(P)H oxidase, urinary LPO excretion and H2O2 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 Ang II did not change its level. 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. Ang II can also cause renal damage via enhanced oxidative stress. NAD(P)H oxidase is the major source of oxidative stress and is stimulated by Ang II in the vascular smooth muscle cells. We have previously demonstrated that NAD(P)H oxidase expression is enhanced in the kidney of the hypertension model, spontaneously hypertensive rats. It is expressed mainly in podocytes, endothelium, distal convoluted tubules and fibroblast. 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 p67phox but also membrane component 22phox. As a result of the enhanced expression of NAD(P)H oxidase, urinary LPO excretion and H2O2 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.

Figure 2. Western blot analysis for NAD(P)H oxidase cytosolic component p47phox in the kidney homogenates. The average 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. 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.
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, arteriolar sclerosis, 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 pathological role in the renal damage of DSHF rats. Not only suppression of renal Ang II by ACEI...
Tojo et al Oxidative Stress in the Kidney With Heart Failure 839


Angiotensin II and Oxidative Stress in Dahl Salt-Sensitive Rat With Heart Failure
Akihiro Tojo, Maristela Lika Onozato, Naohiko Kobayashi, Atsuo Goto, Hiroaki Matsuoka and
Toshiro Fujita

Hypertension. 2002;40:834-839; originally published online October 14, 2002;
doi: 10.1161/01.HYP.0000039506.43589.D5
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/40/6/834

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/