Endothelial Dysfunction and Xanthine Oxidoreductase Activity in Rats With Human Renin and Angiotensinogen Genes


Abstract—We examined whether xanthine oxidoreductase (XOR), a hypoxia-inducible enzyme capable of generating reactive oxygen species, is involved in the onset of angiotensin (Ang) II–induced vascular dysfunction in double-transgenic rats (dTGR) harboring human renin and human angiotensinogen genes. In 7-week-old hypertensive dTGR, the endothelium-mediated relaxation of noradrenaline (NA)-precontracted renal arterial rings to acetylcholine (ACh) in vitro was markedly impaired compared with Sprague Dawley rats. Preincubation with superoxide dismutase (SOD) improved the endothelium-dependent vascular relaxation, indicating that in dTGR, endothelial dysfunction is associated with increased superoxide formation. Preincubation with the XOR inhibitor oxypurinol also improved endothelium-dependent vascular relaxation. The endothelium-independent relaxation to sodium nitroprusside was similar in both strains. In dTGR, serum 8-isoprostaglandin F\textsubscript{2α}, a vasoconstrictor and antinatriuretic arachidonic acid metabolite produced by oxidative stress, was increased by 100%, and the activity of XOR in the kidney was increased by 40%. Urinary nitrate plus nitrite (NO\textsubscript{x}) excretion, a marker of total body NO generation, was decreased by 85%. Contractile responses of renal arteries to Ang II, endothelin-1 (ET-1), and NA were decreased in dTGR, suggesting hypertension-associated generalized changes in the vascular function rather than a receptor-specific desensitization. Valsartan (30 mg/kg PO for 3 weeks) normalized blood pressure, endothelial dysfunction, and the contractile responses to ET-1 and NA. Valsartan also normalized serum 8-isoprostaglandin F\textsubscript{2α} levels, renal XOR activity, and, to a degree, NO\textsubscript{x} excretion. Thus, overproduction of Ang II in dTGR induces pronounced endothelial dysfunction, whereas the sensitivity of vascular smooth muscle cells to nitric oxide is unaltered. Ang II–induced endothelial dysfunction is associated with increased oxidative stress and vascular xanthine oxidase activity. (Hypertension. 2001;37[part 2]:414-418.)

Key Words: endothelium ■ superoxide ■ arachidonic acid ■ xanthine ■ endothelin ■ angiotensin II ■ receptors, angiotensin II

The endothelium regulates vasomotor tone, local hemostasis, and proliferation by releasing vasodilator substances (nitric oxide [NO], prostacyclin, or endothelium-derived hyperpolarizing factor) and vasoconstrictor substances (endothelin-1 [ET-1], angiotensin II [Ang II], thromboxane A\textsubscript{2}, or free radicals) in response to physiological stimuli.1–3 Maintenance of endothelial integrity is critical for the preservation of blood flow and the prevention of thrombosis.4 Ang II may cause oxidative stress in essential hypertension.4 NADPH oxidase is the major source of superoxide in vascular cells and myocytes5 and is induced by Ang II.5–7 Xanthine oxidoreductase (XOR) is a hypoxia-inducible enzyme8 also capable of producing superoxide anion in the vascular endothelium.9,10 Spontaneously hypertensive rats (SHR) and Dahl salt-sensitive rats have higher XOR activities in the kidney and other tissues.11,12 We13 have also demonstrated that chronic inhibition of NO synthase by NG-nitro-L-arginine methyl ester (L-NAME) induces renal XOR in SHR. Hypertension and vascular damage in double-transgenic rats (dTGR) harboring the human renin and human angiotensinogen genes are dependent on local Ang II formation.14,15 Perivascular leukocyte infiltration and endothelial adhesion molecule activation play a central role in the pathogenesis of Ang II–induced myocardial and renal damage in dTGR.16,17 Ang II induces perivascular inflammation and cell proliferation, even by blood pressure–independent
mechanisms. The transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are involved in the initiation of chemokine and cytokine overexpression in dTGR. Clozel et al20 made similar observations on monocyte/macrophage infiltration and endothelial dysfunction in SHR. In the present study, we tested whether increased oxidative stress and XOR activity are involved in the onset of Ang II--induced vascular dysfunction in dTGR.

Methods
We used 40 male dTGR (4 weeks old) and 10 age-matched normotensive Sprague Dawley (SD) rats (Max Delbrück Center for Molecular Medicine). The dTGR are described elsewhere.14,15 The protocols were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland, whose standards correspond to those of the American Physiological Society. The rats had free access to chow (NaCl 0.8%) and drinking water. dTGR and normotensive SD control rats were divided into 3 groups: (1) dTGR control group (n = 25), (2) dTGR plus valsartan group (n = 15), and (3) SD control group (n = 10). Valsartan was given by gavage once a day (30 mg/kg) for 3 weeks. This dose produced the maximal antihypertensive effect in dTGR in our previous study.21 Control dTGR and SD rats received the same volume of vehicle (0.5% carboxymethyl cellulose). Systolic blood pressure was measured weekly by a tail-cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, model 179-2AB, ITIC Life Science) 24 hours after the last drug dose. At 7 weeks, urine was collected over 24 hours; the animals were then decapitated, and blood samples were taken for serum 8-isoprostaglandin F2α measurement. The heart and kidneys were excised, washed with ice-cold saline, blotted dry, and weighed. Tissue samples were snap-frozen in liquid nitrogen, and samples for autoradiography were put in isopentane (−25°C). All samples were stored at −20°C until assayed. For measurements of vascular responses, the renal artery was carefully excised and cleaned of the adherent connective tissue. Two successive 2-mm sections, 3 mm distal from the renal artery-aorta junction, were used. For morphological analysis, tissue samples were fixed in 4% buffered paraformaldehyde at room temperature, was carefully excised and cleaned of the adherent connective tissue.

The endothelium intact arterial rings were placed between stainless steel hooks and mounted in an organ bath chamber in physiological salt solution (pH 7.4) of the following composition (mmol/L): NaCl 119.0, NaHCO3 25.0, glucose 11.1, CaCl2 1.6, KCl 4.7, KH2PO4 1.2, and MgSO4 1.2 aerated with 95% O2 and 5% CO2. The rings were equilibrated for 60 minutes at 37°C with a resting tension of 1.5 mN. Vascular contractions were evoked by a single dose of Ang II (0.1 mmol/L for 60 minutes), respectively. The concentration (0.1 mmol/L) of noradrenaline (NA). The concentration contraction curves to cumulative NA and ET-1 and the concentration relaxation curves to cumulative ACh and sodium nitroprusside were determined. Because of rapid development of tachyphylaxis, Ang II was given as a single concentration (0.1 mmol/L). To evaluate the role of superoxide anion and XOR in the pathogenesis of endothelial dysfunction, relaxation responses to ACh were also examined in dTGR after preincubation with superoxide dismutase (SOD; 750 IU/mL for 45 minutes) and oxyquinol (1 mmol/L for 60 minutes), respectively.

For autoradiographic studies, frozen kidney sections (20 μm thick) were cut on a cryostat at −17°C, thaw-mounted onto Super Frost Plus slides, dried in a dessicator under reduced pressure at 4°C overnight, and stored at −80°C with silica gel until further processing for autoradiographic studies. ACE, angiotensin receptors (AT1 and AT2), and neutral endopeptidase were quantified by in vitro autoradiography as described previously.22–27 Renal AT1 mRNA expression was measured with real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR; ABI’s Prism 7700 Sequence Detection System, Perkin Elmer) according to the instructions of the TaqMan EZ RT-PCR TaqMan probe protocol. The following RT-PCR primers and TaqMan-probe for GAPDH and AT1, receptor were used: GAPDH forward, AAAGTCGTGATCAATGG-GAAAC; GAPDH reverse, ACCCCATTGTG ATACGG; GAPDH probe, CATCACCTCTCCGAGCAGCGGAT; FAM (6-carboxyfluorescein) and TAMRA (quencher) labeled; AT1, receptor forward, CCATCGTCCACCAATGAAG; AT1, receptor reverse, TGCAAGTGACTTGGCCAC; and AT1, receptor probe, FAM-TGCCCTTCCGGCGACGAT-TAMRA.

Renal XOR activity was measured fluorometrically as described in detail previously.12 and urinary nitrate plus nitrite concentration (NOx) was measured with a commercially available nitrate/nitrite colorimetric assay kit (Cayman). Serum 8-isoprostaglandin F2α concentration was determined by ELISA (Cayman) according to the instructions of the manufacturer.

Data are presented as mean±SEM. Statistically significant differences in mean values were tested by ANOVA and the Tukey multiple range test. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time points. The differences were considered significant at P<0.05. Data were analyzed with SYSTAT statistical software.

Results
Valsartan treatment normalized blood pressure, cardiac hypertrophy, and 24-hour proteinuria in dTGR (Table). ACh evoked a dose-dependent relaxation in both strains (Figure 1A). However, endothelium-dependent vascular relaxations to ACh were markedly impaired in dTGR compared with controls (Figure 1A). Maximal relaxation in the renal arteries was 43±13% in dTGR and 94±2% in SD rats, respectively (P<0.05). Valsartan treatment improved vascular relaxation responses to ACh (Figure 1A). Preincubation of the renal arteries with SOD (Figure 1B) and oxyquinol (Figure 1C) markedly improved endothelium-dependent vascular relaxations to ACh in dTGR. In an additional in vitro experiment, we examined the effects of SOD and oxyquinol on endothelium-dependent vascular relaxation in 7-week-old normotensive SD rats (n = 5). Preincubation of the renal arteries with SOD did not markedly influence the endothelium-dependent vascular relaxation to ACh (maximal relaxation 80.0±5.2% versus 72.7±7.2%, P = 0.46). Endothelium-dependent vascular relaxation in response to ACh was also unaltered by preincubation with oxyquinol (80.2±4.2% versus 73.1±8.0%, P = 0.47). Serum 8-isoprostaglandin F2α levels were increased by 100% in dTGR (Figure 1D), and the activity of XOR in the kidney was increased by 40% (Figure 1E), whereas urinary NOx excretion was decreased by 85% (Figure 1F). Valsartan treatment improved vascular relaxation responses to ACh (Figure 1A), normalized serum 8-isoprostaglandin F2α levels (Figure 1D), renal XOR activity (Figure 1E), and, to a degree, NOx excretion (Figure 1F).

Endothelium-independent vascular relaxation to sodium nitroprusside was similar between dTGR and SD rats (Figure 2A). Valsartan improved endothelium-independent vascular relaxation (Figure 2A). Contractions to a single dose of Ang II (0.1 mmol/L) were more pronounced in SD rats than in untreated and valsartan-treated dTGR (Figure 2B). The contractile responses to NA (Figure 2C) and ET-1 (Figure 2D) were also markedly

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impaired in dTGR but were normalized by valsartan. The vascular media-to-lumen ratio tended to be slightly increased in dTGR compared with SD rats (0.20 ± 0.04 versus 0.14 ± 0.09); however, this difference did not reach statistical significance (P = 0.32). The vascular morphology between SD and dTGR was similar by light microscopy.

In dTGR, AT₁ and AT₂ receptor expressions in the cortex and medulla were markedly decreased, whereas ACE expression was unchanged (Table). Valsartan slightly increased cortical AT₁ receptor expression. AT₁ expression in medulla, as well as AT₂ and ACE expressions, was unchanged. In dTGR, renal AT₁ mRNA expression was markedly decreased compared with SD rats (Table). Valsartan treatment increased renal AT₁ mRNA expression to levels found in SD rats.

Discussion
Hypertension, disturbances in renal hemodynamics, and perivascular inflammation in dTGR are due to local Ang II formation in the heart, kidney, and vasculature. Because the endothelium in large part controls vasomotor tone, as well as intrarenal hemodynamics, by releasing endothelium-derived vasoactive substances, we characterized renal vascular functions in dTGR. Furthermore, we tested whether increased oxidative stress and XOR activity are involved in the onset of Ang II–induced vascular dysfunction in dTGR. We found that hypertension in dTGR is associated with impaired endothelium-mediated vascular relaxation, whereas endothelium-independent vascular relaxation in dTGR was unchanged. Ang II–induced endothelial dysfunction was associated with increased superoxide formation and vascular XOR activity, because preincubations of the arteries with SOD and oxypurinol markedly improved ACh-mediated vascular relaxation, respectively. Furthermore, the serum level of 8-isoprostaglandin F₂α, a vasoconstrictor and antinatriuretic arachidonic acid metabolite produced by oxidative stress, was increased by 100% in dTGR, and the activity of XOR in the kidney was increased by 40%. We also demonstrated that urinary NO excretion, a commonly used marker of total body NO generation, was markedly decreased in dTGR. Taken to-
together, our findings indicate that overproduction of Ang II in dTGR induces pronounced endothelial dysfunction, whereas the sensitivity of vascular smooth muscle cells to NO is unaltered. Ang II–induced endothelial dysfunction is associated with increased oxidative stress and vascular xanthine oxidase activity.

Ang II induces pronounced endothelial dysfunction when infused into normotensive rats.7,29 Interestingly, subpressor Ang II doses selectively modify vascular NO bioavailability.29 Moreover, endothelial dysfunction may precede other established Ang II–induced vascular effects.29 Ang II stimulates superoxide generation by increasing the activity of the NAD(P)H oxidase in vitro6 and in vivo.7 Furthermore, endothelial dysfunction in Ang II–infused rats can be corrected by liposome-encapsulated SOD and AT1 receptor blockade.7 Even a subpressor dose of Ang II causes oxidative stress, as measured by the production of 8-isoprostaglandin F2α, a noncyclooxygenase–produced metabolite of arachidonic acid.4,30 In agreement with these findings, we showed here that the serum 8-isoprostaglandin F2α level was ~100% increased in untreated dTGR and that preincubation of the renal arteries with SOD effectively improved ACh-mediated, endothelium-dependent vascular relaxation. Our findings agree with previous studies conducted in other transgenic animal models with increased Ang II production.31–33 Whether or not endothelial dysfunction in dTGR is due to the direct effects of Ang II on endothelial cells and/or Ang II–induced hypertension is capable of impairing endothelial function indirectly remains to be determined. We also demonstrated in the present study that chronic AT1 receptor blockade effectively normalized endothelium-dependent vascular relaxation in dTGR. AT1 receptor blockade also slightly improved the sensitivity of vascular smooth muscle cells to NO.

XOR plays an important role in purine catabolism by producing urate from the ATP degradation products xanthine and hypoxanthine. Previous studies by us and others have shown that SHR and Dahl salt-sensitive rats have higher XOR activities in the kidney and other tissues.11,12 We have also demonstrated that chronic inhibition of NO synthase by L-NAME induces renal XOR in SHR.13 In the present study, preincubation of renal arteries with the XOR inhibitor oxypurinol improved endothelium-dependent vascular relaxation by 20%. Renal XOR activity was also increased substantially in dTGR. Hence, our data support the notion that endothelial dysfunction in dTGR is mediated, at least in part, by reactive oxygen species generated by XOR. However, we would like to emphasize that endothelial dysfunction in dTGR was only partially corrected by XOR inhibition, whereas it was completely normalized by SOD. Therefore, it is very likely that, compared with XOR, other enzyme systems capable of producing reactive oxygen species (ie, NADH/NADPH oxidase and uncoupled endothelial NO synthase) play a more important role in the onset of Ang II–induced vascular dysfunction in dTGR.

In the renal artery, the contractile response to Ang II was less prominent in dTGR than in SD rats. This finding is in line with a previous study by Arnet et al32 using aortic rings from TGR(mRen2)27 rats. We48 reported recently that Ang II concentrations in the plasma and kidney are 4- to 5-fold higher in dTGR than in SD rats. AT1 receptor protein expression and AT1 mRNA were both decreased in dTGR kidneys. Our findings clearly indicate that high circulating Ang II and tissue Ang II concentrations induce agonist-dependent AT1 receptor downregulation in dTGR. Our data thus contradict the recent findings in TGR(mRen2)27 rats, where fulminant hypertension and elevated circulating and tissue Ang II concentrations were associated with markedly increased AT1 receptor expression in renal vasculature and glomeruli.34 We also showed that AT2 receptors are downregulated in dTGR. Hence, our findings indicate that intrarenal AT1 and AT2 receptors are subject to negative feedback regulation.

Previous studies35 have underscored the major importance of the endothelin system in the regulation of kidney hemodynamics and functions. Furthermore, recent data46 suggest that in acute renal failure, endothelial dysfunction is associated with increased circulating and tissue ET-1 levels. Cell culture studies have revealed that Ang II is a powerful stimulator of ET-1 synthesis and release in vascular smooth muscle and endothelial cells. We showed that the vascular contractile responses to ET-1 in dTGR were markedly attenuated in the renal arteries and that chronic AT1 receptor blockade was associated with normalization of ET-1 contractile responses in dTGR. We37 reported recently that the ET-A/ET-B receptor antagonist bosentan markedly ameliorates end-organ dam-

Blood Pressure, Heart Weight, Proteinuria, and Renal Angiotensin Receptors (AT1 and AT2), ACE, and AT1 mRNA Expressions in dTGR, Valsartan-Treated dTGR, and SD Controls

<table>
<thead>
<tr>
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<th>DTGR</th>
<th>DTGR Plus Valsartan</th>
<th>SD</th>
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<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>219±8</td>
<td>122±8*</td>
<td>108±3*</td>
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<tr>
<td>Heart weight (mg) to body weight (g) ratio</td>
<td>5.94±0.35</td>
<td>3.59±0.21*</td>
<td>3.11±0.08*</td>
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<td>Proteinuria, mg/d</td>
<td>82.1±17.8</td>
<td>15.7±2.9*</td>
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<tr>
<td>AT1 receptor expression, DPM/mm²</td>
<td>7.1±1.5</td>
<td>16.5±2.0*</td>
<td>25.1±1.3*</td>
</tr>
<tr>
<td>Cortex</td>
<td>22.6±3.0</td>
<td>24.7±2.8</td>
<td>47.8±1.8*†</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.092±0.061</td>
<td>0.39±0.13</td>
<td>1.02±0.28*</td>
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<tr>
<td>AT2 receptor expression, DPM/mm²</td>
<td>0.15±0.07</td>
<td>0.15±0.06</td>
<td>0.87±0.13†</td>
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<tr>
<td>Cortex</td>
<td>167.4±12.3</td>
<td>136.5±6.9</td>
<td>151.8±12.1</td>
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<tr>
<td>Medulla</td>
<td>84.4±8.0</td>
<td>338.3±8.0*</td>
<td>294.1±42.9*</td>
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<tr>
<td>ACE expression, DPM/mm²</td>
<td>12.1</td>
<td>8.0*</td>
<td>29.4±2.9</td>
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<tr>
<td>AT1 mRNA expression</td>
<td>12.3</td>
<td>9.0*</td>
<td>33.6±2.9</td>
</tr>
</tbody>
</table>

*P<0.05 vs dTGR, †P<0.05 vs valsartan-treated dTGR. Values are mean±SEM (n=6 to 10).

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age in dTGR, which supports these findings. Interestingly, vascular contractile responses to NA were also attenuated in dTGR and were normalized by valsartan treatment. Previous studies have shown that Ang II stimulates sympathetic nervous system centrally and peripherally through activation of the presynaptic AT1 receptors. Taken together, our findings of the diminished vascular contractile responses to Ang II, ET-1, and NA in dTGR support generalized vascular changes associated with hypertension rather than a receptor-specific desensitization.

In conclusion, our findings indicate that overproduction of Ang II in dTGR induces pronounced endothelial dysfunction, whereas the sensitivity of vascular smooth muscle cells to NO is unaltered. Ang II–induced endothelial dysfunction is associated with increased oxidative stress and vascular xanthine oxidase activity.

Acknowledgments

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References

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