Endothelial Dysfunction and Salt-Sensitive Hypertension in Spontaneously Diabetic Goto-Kakizaki Rats

Zhong Jian Cheng, Timo Vaskonen, Ilkka Tikkanen, Kaisa Nurminen, Heikki Ruskoaho, Heikki Vapaatalo, Dominik Muller, Joon-Keun Park, Friedrich C. Luft, Eero M.A. Mervaala

Abstract—Endothelial dysfunction is associated with hypertension, hypercholesterolemia, and heart failure. We tested the hypothesis that spontaneously diabetic Goto-Kakizaki (GK) rats, a model for type 2 diabetes, exhibit endothelial dysfunction. Rats also received a high-sodium diet (6% NaCl [wt/wt]) and chronic angiotensin type 1 (AT1) receptor blockade (10 mg/kg PO valsartan for 8 weeks). Compared with age-matched nondiabetic Wistar control rats, GK rats had higher blood glucose levels (9.3±0.5 versus 6.9±0.2 mmol/L for control rats), 2.7-fold higher serum insulin levels, and impaired glucose tolerance (all P<0.05). Telemetry-measured mean blood pressure was 15 mm Hg higher in GK rats (P<0.01) compared with control rats, whereas heart rates were not different. Heart weight–to–body weight ratios were higher in GK rats (P<0.05), and 24-hour albuminuria was increased 50%. Endothelium-mediated relaxation of noradrenaline-precontracted mesenteric arterial rings by acetylcholine was impaired compared with the control condition (P<0.05), whereas the sodium nitroprusside–induced relaxation was similar. Preincubation of the arterial rings with the NO synthase inhibitor NG-nitro-L-arginine methyl ester and the cyclooxygenase inhibitor diclofenac inhibited relaxations to acetylcholine almost completely in GK rats but not in Wistar rats, suggesting that endothelial dysfunction can be in part attributed to reduced relaxation via arterial K+ channels. Perivascular monocyte/macrophage infiltration and intercellular adhesion molecule-1 overexpression were observed in GK rat kidneys. A high-sodium diet increased blood pressure by 24 mm Hg and 24-hour albuminuria by 350%, induced cardiac hypertrophy, impaired endothelium-dependent relaxation further, and aggravated inflammation (all P<0.05). The serum level of 8-isoprostaglandin F2α, a vasoconstrictor and antinatriuretic arachidonic acid metabolite produced by oxidative stress, was increased 400% in GK rats on a high-sodium diet. Valsartan decreased blood pressure in rats fed a low-sodium diet and prevented the inflammatory response. In rats fed a high-sodium diet, valsartan did not decrease blood pressure or improve endothelial dysfunction but protected against albuminuria, inflammation, and oxidative stress. As measured by quantitative autoradiography, AT1 receptor expression in the medulla was decreased in GK compared with Wistar rats, whereas cortical AT1 receptor expression, medullary and cortical angiotensin type 2 (AT2) receptor expressions, and adrenal ACE and neutral endopeptidase expressions were unchanged. A high-sodium diet did not influence renal AT1, AT2, ACE, or neutral endopeptidase expressions. In valsartan-treated GK rats, the cortical and medullary AT1 receptor expressions were decreased in the presence and absence of a high-sodium diet. A high-sodium diet increased plasma brain natriuretic peptide concentrations in presence and absence of valsartan treatment. We conclude that hypertension in GK rats is salt sensitive and associated with endothelial dysfunction and perivascular inflammation. AT1 receptor blockade ameliorates inflammation during a low-sodium diet and partially protects against salt-induced vascular damage by blood pressure–independent mechanisms. (Hypertension. 2001;37[part 2]:433-439.)

Key Words: acetylcholine ■ nitroprusside ■ receptors, angiotensin ■ angiotensin-converting enzyme ■ peptides

Non–insulin-dependent diabetes mellitus (NIDDM) in humans is a major risk factor for end-stage renal disease1 and is commonly accompanied by hypertension, dyslipidemia, and prothrombotic factors.2 In fact, frank hypertension is frequently found in subjects who have insulin resistance but not NIDDM, and ~80% of patients with NIDDM are hypertensive at the time of the diagnosis.2 At present, the mechanisms involved in the pathogenesis of hypertension and vascular complications in NIDDM are poorly understood.
Several lines of evidence suggest a crucial role of oxidative stress and angiotensin II (Ang II) in the pathogenesis of hypertension and endothelial dysfunction.\textsuperscript{3,4} Endothelial dysfunction in hypertension may be due to impaired NO synthesis and/or inactivation of endothelium-derived NO by reactive oxygen species, such as superoxide, hydrogen peroxide, and peroxynitrite.\textsuperscript{3} The balance between NO and superoxide might be even more important than the absolute levels of either alone.\textsuperscript{3} We have recently shown in double transgenic rats harboring human renin and human angiotensinogen genes that Ang II induces profound inflammation and activates redox-sensitive genes via nuclear factor-kB activation, independent of blood pressure.\textsuperscript{5–7}

Endothelial dysfunction and oxidative stress have also been linked to the pathogenesis of diabetes.\textsuperscript{8,9} Impaired vascular relaxation in response to acetylcholine (ACh) has been reported in diabetic animals and humans.\textsuperscript{8,9} The mechanisms of endothelial dysfunction are incompletely understood; increased vascular production of superoxide\textsuperscript{8} and inactivation of endothelium-derived relaxing factors by glycosylated hemoglobin\textsuperscript{8,10} have been suggested. Interestingly, high glucose concentration and hyperglycemia promote leukocyte adhesion to the endothelium through upregulation of adhesion molecule expression in a nuclear factor-kB-dependent fashion.\textsuperscript{11} Whether increased adhesion of leukocytes to the endothelium causes endothelial dysfunction in NIDDM is not known. The pathogenesis of NIDDM in Goto-Kakizaki (GK) rats, a nonobese type II diabetic rat model originally derived by repeated inbreeding of glucose-intolerant Wistar rats,\textsuperscript{12} includes impaired ontogenetic development of islet cells, abnormal insulin release after a glucose load, insulin resistance, a basal hyperinsulinemia, and abnormal glucose metabolism.\textsuperscript{13,14} Recent studies have demonstrated that GK rats showed several structural changes in the kidney similar to those observed in NIDDM patients without overt kidney disease. These changes consisted of thickening of the glomerular basement membrane and tubular basement membrane, glomerular hypertrophy, and early podocyte damage.\textsuperscript{13–15} However, the kidneys of GK rats do not typically show glomerulosclerosis or interstitial fibrosis.\textsuperscript{13–15} We used GK rats to test whether endothelial dysfunction and inflammation participate in the pathogenesis of hypertension and diabetic nephropathy in GK rats. We also evaluated the cardiovascular and renal effects of a high-sodium diet and chronic angiotensin type 1 (AT\textsubscript{1}) receptor blockade in GK rats because of the potential importance of this system.\textsuperscript{16}

Methods

We used forty 8-week-old male spontaneously diabetic GK rats (M&B, Ejby, Denmark) and 10 age-matched nondiabetic Wistar rats. The protocols were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Helsinki, Finland, whose standards correspond to those of the American Physiological Society. In the beginning of the study, blood pressure and body weight–matched GK rats were divided into 4 groups (n=10 in each group) that received different diet and drug regimens for 8 weeks: group 1, GK control rats on a low-sodium diet (NaCl 0.8% [wt/wt]); group 2, GK rats on a high-sodium diet (NaCl 6% [wt/wt]); group 3, GK rats treated with valsartan (10 mg/kg mixed in the food); and group 4, GK rats on a high-sodium diet plus valsartan treatment. Wistar rats served as controls. The rats had free access to chow and drinking water. Systolic blood pressure was measured by using a tail-cuff blood pressure analyzer (Applio-2AB Blood Pressure Analyzer, model 179-2AB, IITC Life Science)\textsuperscript{17} or by radiotelemetry (Dataquest IV telemetry system, Data Sciences International).\textsuperscript{18} At 16 weeks, an oral glucose tolerance test was performed in some overnight-fasted GK and Wistar rats (1 g/kg glucose by gavage).\textsuperscript{19} At the end of the experimental period, urine samples were collected over 24 hours, and the animals were then decapitated, and blood samples were taken for blood glucose, plasma atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), serum insulin, and serum 8-isoprostaglandin F\textsubscript{2a} measurements. The heart and kidneys were excised, washed with ice-cold saline, blotted dry, and weighed. Tissue samples for immunohistochemistry and autoradiography were snap-frozen in isopentane (−35°C). All samples were stored at −80°C until they were assayed. For measurements of vascular responses, superior mesenteric arteries were carefully excised and cleaned of the adherent connective tissue. Two successive sections (3 mm), 5 mm distal from the mesenteric artery–aorta junction, were used. For morphological analysis, tissue samples were fixed in 4% buffered paraformaldehyde at room temperature, dehydrated in graded alcohol, and embedded in paraffin.\textsuperscript{20} Sections (2 to 3 μm) were cut with the use of a microtome (Leitz 1512). The sections were deparaffinized and rehydrated before being stained with hematoxylin-eosin and Masson’s trichrome. The tissues were examined without knowledge of the rat group from which they were taken.

Frozen kidneys were processed, and semiquantitative scoring was performed as described in detail previously.\textsuperscript{2,20} Briefly, ice-cold acetone-fixed cryosections (6 μm) were air-dried and immersed in TBS (0.05 mol/L Tris buffer and 0.15 mol/L NaCl, pH 7.6). All incubations were performed in a humid chamber at room temperature, dehydrated in graded alcohol, and embedded in paraffin.\textsuperscript{20} Sections (2 to 3 μm) were cut with the use of a microtome (Leitz 1512). The sections were deparaffinized and rehydrated before being stained with hematoxylin-eosin and Masson’s trichrome. The tissues were examined without knowledge of the rat group from which they were taken.

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The relaxation curves to cumulative ACh and sodium nitroprusside (SNP) were determined in mesenteric artery rings precontracted with 1 μmol/L norepinephrine (FTO3C transducer, model 7 C8 polygraph, Grass Instrument Co) as described earlier.\textsuperscript{21} To analyze the components of endothelium-dependent vascular relaxation, cyclooxygenase and/or NO synthase (NOS) were inhibited in some experiments with diclofenac (3 mol/L) and N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME, 0.1 mmol/L), 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 desiccator under reduced pressure at 4°C overnight, and stored at −80°C with silica gel until further processing for autoradiographic studies. ACE, angiotensin receptors types 1 and 2 (AT\textsubscript{1} and AT\textsubscript{2}, respectively), and neutral endopeptidase (NEP) were quantified by in vitro autoradiography as described earlier.\textsuperscript{22–27}

Urinary albumin was measured by ELISA with rat albumin used as a standard (Immun Diagnostik), and urinary nitrate+nitrite (NO\textsubscript{X}) concentration was assessed with a commercially available NO\textsubscript{X} colorimetric assay kit (Cayman). Serum 8-isoprostaglandin F\textsubscript{2a} concentration was determined by ELISA (Cayman), and serum insulin was determined by radiommunoassay (Linco Research) according the instructions of the manufacturer. Blood glucose was measured with an automatic analyzer (Reflotron, Boehringer-
Mannheim). Plasma ANP and BNP levels were measured by radioimmunoassay as described in detail earlier.28 Data are presented as mean ± SEM. Statistically significant differences in mean values were tested by ANOVA and the Fisher protected least significant difference test. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time points. The differences were considered significant at \( P < 0.05 \). The data were analyzed by use of SYSTAT statistical software (SYSTAT Inc).

### Results

Radiotelemetry mean arterial blood pressure (MAP) was 15 mm Hg higher in 16-week-old GK rats than in control rats (Figure 1A); no difference in heart rate was observed (data not shown). MAP displayed a 24-hour rhythm characterized by several nighttime peaks, the first of which occurred immediately at the start of the dark period. GK rats showed impaired glucose tolerance in an oral glucose tolerance test (Figure 1B). Systolic blood pressure, heart weight–to–body weight ratios, and 24-hour albuminuria were higher in GK rats compared with Wistar control rats (Figure 1D to 1F). Valsartan decreased blood pressure moderately during the low-sodium diet but did not significantly change heart and kidney weights or albuminuria (Figure 1C to 1F). During the high-sodium diet, valsartan did not decrease blood pressure or prevent the development of cardiac hypertrophy, but it significantly decreased albuminuria (Figure 1C to 1F).

Endothelium-mediated vascular relaxation of norepinephrine-precontracted mesenteric arterial rings in response to ACh was markedly impaired in GK rats compared with Wistar rats, but the endothelium-independent relaxations to SNP were similar in both strains (Figure 2A and 2B). Preincubation of the arterial rings with the NOS inhibitor L-NAME and the cyclooxygenase inhibitor diclofenac inhibited relaxations to ACh most completely in GK rats but not in Wistar rats (Figure 3). The high-sodium diet impaired vascular relaxation in response to ACh and also to SNP (Figure 2A and 2B). Valsartan treatment improved endothelium-dependent vascular relaxation during the low-sodium diet.
Vessels were precontracted with 1 mM preincubation with NOS inhibitor L-NAME and cyclooxygenase inhibitor diclofenac. Serum 8-isoprostaglandin F_2alpha levels tended (P<0.05) to be higher in GK rats than in Wistar rats (Figure 2C). The high-sodium diet increased P, levels tended (P<0.05 vs Wistar rats) to be higher in GK rats on low-sodium diet. The improvement of endothelium-dependent vascular relaxation by valsartan was also resistant to NOS and cyclooxygenase inhibition (Figure 2A and 2B). Endothelium-dependent vascular relaxation resistant to NOS and cyclooxygenase inhibition was impaired in GK rats (P<0.05 vs Wistar rats). High-sodium diet impaired endothelium-dependent vascular relaxation resistant to NOS and cyclooxygenase inhibition (P<0.05 vs GK rats on low-sodium diet). Valsartan improved vascular relaxation during low-sodium diet (P<0.05 vs GK rats on low-sodium diet). Symbols indicate mean±SEM (n=6 to 10 in each group, 1 vascular ring per animal).

The kidneys of GK rats on the high-sodium diet showed some mesangial thickening and slight thickening of the media of the afferent arterioles. There were no clear signs of glomerulosclerosis or interstitial fibrosis (Figure 4A). In GK rats on the low-sodium diet, there was already a significant monocyte/macrophage infiltration (ED-1-positive cells) in the renal perivascular space and increased expression of ICAM-1 in the interstitium, intima, and adventitia of the small renal vessels (Figure 4D and 4E). The high-sodium diet aggravated the inflammatory response in GK rats (Figure 4B to 4E). Valsartan treatment decreased the number of ED-1-positive cells and ICAM-1 expression during both low- and high-sodium diets.

**AT1 receptor expression in the medulla was decreased in GK rats compared with Wistar rats, whereas cortical AT1 receptor expression, medullary and cortical AT2 receptor expressions, and renal ACE and NEP expressions were unchanged (Table).** The high-sodium diet did not influence renal AT1, AT2, ACE, or NEP expressions. In valsartan-treated GK rats, the cortical and medullary AT1 receptor expressions were decreased in the presence and absence of the high-sodium diet. There were no differences between the groups in plasma ANP concentrations (Table). Plasma BNP levels were increased by the high-sodium diet in the presence and absence of valsartan treatment (Table). Blood glucose and serum insulin levels were higher in GK rats compared with Wistar rats but were not markedly influenced by the high-sodium diet or valsartan treatment (Table).

### Renal AT1 and AT2 Receptor, ACE, and NEP Expression and ANP, BNP, Blood Glucose, and Serum Insulin Levels in Spontaneously Diabetic GK Rats and Nondiabetic Wistar Rats After 8 Weeks on Different Diet and Drug Regimens

<table>
<thead>
<tr>
<th></th>
<th>GK Rats on Low-Sodium Diet</th>
<th>GK Rats on High-Sodium Diet</th>
<th>GK Rats on Low-Sodium Diet + Valsartan</th>
<th>GK Rats on High-Sodium Diet + Valsartan</th>
<th>Wistar Rats on Low-Sodium Diet</th>
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<tr>
<td>AT1 receptor expression</td>
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<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td>22.8±1.6*†</td>
<td>20.4±1.6 ††</td>
<td>30.4±1.7</td>
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<td>Medulla</td>
<td>42.2±3.5*</td>
<td>51.3±5.1</td>
<td>29.4±2.8*††</td>
<td>27.4±2.2 ††</td>
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<td>0.44±0.19</td>
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<td>Medulla</td>
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<td>ACE expression</td>
<td>209.5±7.0</td>
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<td>206.8±9.5</td>
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<td>221.0±3.5</td>
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<td>NEP expression</td>
<td>378.7±26.4</td>
<td>310.3±27.1</td>
<td>301.0±22.4</td>
<td>300.5±20.3</td>
<td>335.9±17.8</td>
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<td>Plasma ANP, pg/mL</td>
<td>266±36</td>
<td>229±22</td>
<td>234±50</td>
<td>318±53</td>
<td>357±40</td>
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<tr>
<td>Plasma BNP, pmol/L</td>
<td>31.1±2.1</td>
<td>36.4±2.7*</td>
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<td>36.8±4.1*</td>
<td>25.6±0.7</td>
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<td>Blood glucose, mmol/L</td>
<td>9.3±0.5*</td>
<td>10.5±0.8*</td>
<td>8.2±0.3‡</td>
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<td>6.9±0.2</td>
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<tr>
<td>Serum insulin, ng/mL</td>
<td>8.1±1.1*</td>
<td>6.3±0.7</td>
<td>8.3±1.0*</td>
<td>5.4±0.8</td>
<td>3.0±0.7</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P<0.05 vs Wistar rats; †P<0.05 vs GK rats on low-sodium diet; and ‡P<0.05 vs GK rats on high-sodium diet.
## Discussion

The main finding of the present study was that GK rats were already moderately hypertensive on the low-sodium diet and showed marked impairment of endothelium-mediated vascular relaxation. Furthermore, a pronounced perivascular monocyte/macrophage infiltration and adhesion molecule overexpression in the kidneys were observed. We were also able to demonstrate that hypertension in GK rats is strongly dependent on the level of dietary salt. The high-sodium diet also induced marked end-organ damage and aggravated endothelial dysfunction and the inflammatory response associated with increased oxidative stress. Finally, our findings of the beneficial cardiovascular and renal effects of valsartan indicate the involvement of increased renin-angiotensin system activity in the pathogenesis of diabetic nephropathy and vascular complications in GK rats.

Previous studies have revealed that unlike several other rodent models of NIDDM, GK rats are not hypertensive, hyperlipemic, or obese. Furthermore, the prolonged hyperglycemia and hyperinsulinemia in GK rats are not associated with any marked renal functional changes, although structural changes such as thickening of the glomerular and tubular basement membranes as well as glomerular hypertrophy are detected. In good accordance with these findings, we found only a 1.5-fold increase in 24-hour albuminuria in GK rats on the low-sodium diet. However, with radiotelemetry, we were able to detect a 15 mm Hg difference in MAP between GK and Wistar rats. Although hypertension is commonly associated with endothelial dysfunction, it is likely that factors other than hypertension are also involved in the pathogenesis of endothelial dysfunction in GK rats on a low-sodium diet. The endothelium-independent vascular re-

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**Figure 4.** Representative photomicrograph from kidneys of GK rat on high-sodium diet (A) and immunohistochemical photomicrographs of monocyte/macrophage infiltration (ED-1–positive cells) (B) and ICAM-1 expression (C). Semiquantitative scoring of ED-1–positive cells in the kidney is shown in panel D. Fifteen different areas of each sample were analyzed by using a computerized cell count program. Semiquantitative scoring of ICAM-1 expression (from 0 to 5) in the kidney is shown in panel E. Each bar represents n=5. Mean±SEM values are given. *P*<0.05 vs Wis; #P<0.05 vs GK; and α P<0.05 vs GK+Na.
laxation in response to SNP was unchanged in GK rats, indicating that the sensitivity of arterial smooth muscle cells to NO is unaltered.

We also found a profound perivascular monocyte/macrophage infiltration and ICAM-1 overexpression in the kidneys of GK rats on a low-sodium diet. Our finding supports the previous notion that oxygen-derived free radicals generated by the inflammatory cells are able to reduce the bioavailability of endothelium-derived NO.\textsuperscript{3,4} There was no difference in the NO\textsubscript{x} excretion, suggesting that endothelial NO production is unaltered in GK rats. The efficacy of valsartan to ameliorate inflammatory response as well as endothelial dysfunction indicates the involvement of increased renin-angiotensin system activity in the pathogenesis. Our findings agree with earlier studies demonstrating that Ang II stimulates superoxide generation by increasing the activity of NAD(P)H oxidase in vitro\textsuperscript{29} and in vivo.\textsuperscript{30} However, we would like to emphasize that the vascular responses and the degree were analyzed from different tissues, i.e., from the mesenteric artery and the kidney, and in fact, the serum 8-isoprostaglandin level tended to be increased only in GK rats on a low-sodium diet. Therefore, we cannot completely conclude that the endothelial dysfunction found in the present study was due to increased oxidative stress.

The endothelium-mediated relaxation that remains resistant to NOS and cyclooxygenase inhibition is thought to be mediated by endothelium-derived hyperpolarizing factor, which appears to be a cytochrome P450-derived arachidonic acid epoxide. Current data support the concept that the vascular relaxation elicited by endothelium-derived hyperpolarizing factor is mediated by the opening of Ca\textsuperscript{2+}-derived K\textsuperscript{+} channels in the vascular smooth muscle cells. Our findings suggest that endothelial dysfunction in GK rats could be attributed, at least in part, to reduced relaxation via arterial K\textsuperscript{+} channels. Furthermore, our finding that valsartan was able to improve the endothelium-dependent vascular relaxation also after preincubation of the arterial rings with the NOS inhibitor L-NAME and the cyclooxygenase inhibitor diclofenac after preincubation of the arterial rings with the NOS inhibitor l-NAME and the cyclooxygenase inhibitor diclofenac suggests that valsartan is able to improve vascular relaxation via arterial K\textsuperscript{+} channels. However, the improvement of endothelium-dependent vascular relaxation by valsartan might also have been due, at least in part, to stimulation of AT\textsubscript{1} receptors, leading to increased production of bradykinin and NO as demonstrated by Siragy et al.\textsuperscript{31}

High-sodium intake may increase blood pressure, induce cardiac hypertrophy, and deteriorate the therapeutic effects of most antihypertensive drugs.\textsuperscript{32} The effects are mediated primarily by volume overload as well as by increased sympathetic nervous system activity.\textsuperscript{33} Previous studies have demonstrated that hypertension in patients with diabetes is often volume dependent and salt sensitive.\textsuperscript{1} We found that a high-sodium diet causes a marked increase in blood pressure associated with the development of cardiac hypertrophy. A high-sodium diet also induces renal damage and aggravates inflammation and endothelial dysfunction. Furthermore, a high-sodium diet impairs the sensitivity of arterial smooth muscle cells to NO. The mechanism of the impaired sensitivity remains unsolved. The detrimental effects of a high-sodium diet are mediated partly by volume overload, inasmuch as plasma BNP concentration was markedly increased by the high-sodium diet. The 4-fold increase in serum 8-isoprostaglandin F\textsubscript{2a} levels indicates that the detrimental effects of dietary sodium are associated with increased oxidative stress. In contrast, we were unable to detect any salt-induced changes in renal AT\textsubscript{1}, AT\textsubscript{2}, or ACE expressions. The renal expression of NEP, the major metabolizing enzyme for ANP and BNP, was also unaltered by the high-sodium diet. Interestingly, valsartan effectively protected against the development of salt-induced renal damage, inflammatory response, and oxidative stress. Our findings indicate that hypertension in diabetic GK rats is salt sensitive and associated with endothelial dysfunction and perivascular inflammation. AT\textsubscript{1} receptor blockade partially protects against salt-induced vascular damage by blood pressure-independent mechanisms.

Acknowledgments

The study was supported by grants from the Finnish Foundation for Cardiovascular Research, the Academy of Finland, and the Sigrid Jusélius Foundation and by a Helsinki University Central Hospital research grant. The gift of valsartan by Novartis, Basel, Switzerland, is gratefully acknowledged. We are grateful to Dr Piet Finkenberg for histological photomicrographs and Anneli von Behr, Terhi Ilomäki, Tuula Riitihämäki, Toini Sisikonen, and Sari Laakkonen for expert technical assistance.

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Hypertension, 2001;37:433-439
doi: 10.1161/01.HYP.37.2.433
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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