Role of Nitric Oxide–Producing and –Degrading Pathways in Coronary Endothelial Dysfunction in Chronic Kidney Disease

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Cardiovascular events are accelerated in chronic kidney disease (CKD). Although deranged nitric oxide (NO) pathways and asymmetric dimethylarginine (ADMA) cause endothelial dysfunction, no direct evidence for coronary artery endothelial dysfunction in CKD has been documented. CKD was induced in male dogs by heminephrectomy (1/2Nx) or five-sixths nephrectomy (5/6Nx). After 4 wk, renal ablation reduced GFR (control 76 [54 to 85]; 1/2Nx 38 [29 to 47]; 5/6Nx 15 [12 to 46] ml/min) and elevated plasma ADMA (control 1.88 [1.68 to 2.54]; 1/2Nx 2.51 [2.11 to 3.55]; 5/6Nx 3.84 [2.16 to 3.95] μmol/L). Coronary circulatory responses to acetylcholine revealed marked increases in coronary blood flow in control group (83 ± 17% increment) but blunted responses in 1/2Nx (34 ± 8% increment) and 5/6Nx (20 ± 4% increment). The acetylcholine-induced changes in epicardial arteriolar diameter, using needle-lens probe charge-coupled device videomicroscopy, showed similar results. The responsiveness to sodium nitroprusside did not differ among three groups. Plasma nitrite/nitrate levels decreased in 1/2Nx and 5/6Nx, and the mRNA expressions of dimethylarginine dimethylaminohydrolase-II (DDAH-II), ADMA-degradating enzyme, and endothelial NO synthase (eNOS) in coronary arteries were downregulated in 1/2Nx and 5/6Nx. Finally, 4-wk treatment with all-trans retinoic acid restored the impaired endothelium-dependent vasodilation and reversed the expression of eNOS but not DDAH-II. Coronary endothelial function is impaired in the early stage of CKD. The dysfunction is attributed to the downregulation of eNOS and/or DDAH-II in coronary arteries. Furthermore, the manipulation of NO pathways may constitute a therapeutic strategy for the prevention of coronary dysfunction in CKD.

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here is a growing body of evidence that several cardiovascular risk factors reside in chronic kidney disease (CKD) (1,2), and the incidence of cardiovascular events increases even in the stage of apparent macro-/ (3) or microalbuminuria (4). Notably, endothelial dysfunction is established as a predictor for cardiovascular outcomes (5,6), and the impaired endothelium-dependent vasodilation is observed in patients who have CKD and are on maintenance hemodialysis (7). Although endothelial injury/dysfunction constitutes a critical determinant of atherosclerosis through multiple processes, including lipid accumulation, smooth muscle proliferation, and thrombosis (8), whether the progression of CKD impairs the endothelial function in the coronary artery has not been elucidated.

One of the most important functions of the endothelium is a source of various vasoactive substances. Nitric oxide (NO) is a potent vasodilator that is produced by endothelial NO synthase (eNOS) and is deemed an antiatherogenic substance. NO-dependent vasodilation is impaired in several diseases that are associated with atherosclerosis, which may contribute to the progression of atherosclerosis (9). Although the mechanisms of endothelial dysfunction are multifactorial, one contributing abnormality is decreased expression and/or activity of eNOS in the vascular tissue (10,11). Recent studies have demonstrated that the increased level of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, contributes to the development of endothelial dysfunction in various diseases (12). Because its levels are increased in patients with CKD, it is surmised that plasma ADMA levels are predictive of future cardiovascular events and overall mortality in CKD (13). Indeed, in patients with CKD stages 1 through 4, ADMA is well correlated with cardiovascular events and survival (14,15). Finally, the short-term administration of ADMA causes decreased cardiac output and elevated systemic vascular resistance (16,17). Of note, ADMA is metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which was shown recently to have two isoforms, DDAH-I and DDAH-II, and different tissue distribution and regulation for each isoform (18). Furthermore, whereas symmetric dimethylarginine (SDMA), an enantiomer of ADMA, is eliminated almost exclusively by urinary excretion, ADMA is partly eliminated by urinary excretion and extensively metabolized by DDAH (16,19). It is possible, therefore, that DDAH constitutes

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a crucial determinant of plasma ADMA levels and contributes to the pathogenesis of endothelial dysfunction in CKD.

In this study, we investigated the role of NO and ADMA in mediating the endothelial dysfunction of the coronary artery in dogs with various degrees of CKD, using an intravital needle-lens charge-coupled device videomicroscopy (20). Furthermore, we examined whether the expressions of eNOS and DDAH in the coronary artery were altered. We demonstrated that coronary endothelial dysfunction developed even in the early stage of CKD. Furthermore, the expression levels of both eNOS and DDAH-II in the coronary vessels were downregulated in CKD. These studies endorse the important role of the deranged regulation of eNOS and DDAH/ADMA pathways in the initiation of cardiovascular abnormalities in CKD. Finally, the manipulation of the NO pathway and ADMA could constitute a therapeutic strategy for the prevention of cardiovascular events in CKD.

Materials and Methods

Animal Preparation

Adult male hybrid dogs (American foxhounds, Labrador retrievers, and beagles, 16 to 20 kg) were fed a standard diet (80 mmol/d sodium, 20 g/d nitrogen; Oriental Yeast Co., Tokyo, Japan) and had free access to tap water at all times. A Tygon tube was inserted into the abdominal aorta through the right femoral artery for measurement of mean arterial pressure and heart rate under the conscious condition (21). CKD was induced in dogs by heminephrectomy (1/2Nx group; \( n = 8 \)) or by the ligation of two or three branches of the left renal artery and the subsequent contralateral nephrectomy (five-sixths nephrectomy [5/6Nx] group; \( n = 10 \)). Four weeks after the operation, each dog was used for experiment. Sham-operated dogs were used for normal control (\( n = 8 \)). In additional experiments, all-trans retinoic acid (atRA; 2 mg/kg per d orally), which was recently reported to increase NO synthesis in endothelial cells (22), was administered to 5/6Nx dogs for 4 wk, and the same experimental procedures were conducted (control \( n = 7 \); 5/6Nx \( n = 5 \); 5/6Nx+atRA \( n = 7 \)).

For the evaluation of the coronary vascular responsiveness and cardiac function, dogs were anesthetized with sodium pentobarbital (25 mg/kg) and were under the control of a ventilator. A thoracotomy was performed in the left fifth intercostal space, and the heart was suspended in a pericardial cradle. A 6-Fr pigtail catheter (Goodtec, Gifu, Japan) was inserted into the left ventricular (LV) cavity through the right carotid artery to measure LV pressure (LVP). The positive first derivative of LVP was obtained to assess the cardiac contractility.

For measurement of renal plasma flow (RPF) and GFR, 7-Fr balloon catheters (Create Medic, Yokohama, Japan) were temporarily inserted in the bladder through the urethra for urine collection. Bolus injection of 20% p-aminohippurate (200 mg) and 25% inulin (1200 mg) was followed by a continuous infusion of p-aminohippurate (3 mg/min) and inulin (12 mg/min) at a rate of 1.5 ml/min. The infusion was started 90 min before the experiments to obtain stable blood levels of each agent. All experimental procedures were conducted according to the guideline of the Animal Care Committee of Keio University.

Experimental Protocols

After a 30-min stabilization period, acetylcholine (ACh; 0.001 to 0.1 \( \mu g/kg \) per min; Sigma, St. Louis, MO) or sodium nitroprusside (SNP; 0.01 to 1 \( \mu g/kg \) per min; Maruishi Seiyaku, Tokyo, Japan) was infused directly into the left circumflex coronary artery. Coronary blood flow (CBF) was measured with an ultrasonic flowmeter (Transonic Systems, New York, NY) placed at the proximal portion of the left circumflex coronary artery (20). The changes in CBF after 5 min of the commencement of each drug administration were evaluated.

End-diastolic diameters of epicardial coronary arterioles were measured with intravital needle-lens charge-coupled device videomicroscopy (Nihon Kohden, Tokyo, Japan) (20). The needle-lens probe was enclosed in a sheath with a doughnut-shaped balloon on the tip to avoid direct compression of the arteriole by the needle tip, and the tip of the probe was placed gently on the epicardial surface. A green filter was applied to contrast the image of the vessel with the surrounding tissue. Images were converted into black and white video signals and were analyzed by a computer (840AV; Apple Computer, Cupertino, CA) with a freeze-frame modality (20).

Biochemical Analyses

Plasma triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol, and glucose were measured with the enzymatic colorimetric assay. Insulin levels were measured with the immunoradiometric assay method (Abbot Japan, Tokyo, Japan). Plasma C-reactive protein, total plasminogen activator inhibitor-1, and von Willebrand factor were determined by ELISA. Plasma concentrations of homocysteine, t-arginine, ADMA, and SDMA were determined by HPLC (23). Plasma nitrite/nitrate (NOx) concentrations were evaluated using the Griess reaction (24).

mRNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA was isolated from the dog coronary artery with Trizol Reagent (Invitrogen, Carlsbad, CA). Total RNA (50 ng) was reverse-transcribed for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen) for the quantification of mRNA expression. Real-time PCR was performed using the ABI PRISM-7700 sequence detector (PE Applied Biosystems, Foster City, CA) with SYBER Green I Dye (PE Applied Biosystems) for the detection of PCR reaction. The sequences that were used for PCR were as follows: DDAH-I 5'-catgcggcgaatcactaatg-3' for forward primer and 5'-tgagttggcataggggtgc-3' for reverse primer; DDAH-II 5'-cagctgctgactgccttt-3' for forward primer and 5'-agttggcagccggaatcc-3' for reverse primer; eNOS 5'-cagccacagatgtagctgg-3' for forward primer and 5'-actggactcttctcggc-3' for reverse primer; and 18S rRNA 5'-ggttgatcctgc-3' for forward primer and 5'-gccgtggtacttagatg-3' for reverse primer.

Immunohistochemistry

The protein expressions of eNOS, DDAH-I, and DDAH-II in the coronary artery were examined by immunohistochemistry. Tissue sections were incubated with primary antibodies against eNOS (Transduction Laboratories, Lexington, KY) and DDAH-I and II (Abcam, Cambridge, UK) at dilution 1:200 in 5% nonfat milk in PBS for 2 h at 37°C for primary antibodies. After washing with PBS, tissue sections were incubated with secondary anti-goat IgG antibodies conjugated with FITC (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50 dilution. For negative control, the same procedure was performed except for the addition of primary antibodies. Immunolabeled sections were examined with confocal laser scanning microscopy (LSM-510; Carl Zeiss, Oberkochen, Germany) at an excitation wavelength of 488 nm.

Statistical Analyses

Data are expressed as median (range) or mean ± SEM. Results are analyzed by one-way/two-way ANOVA, as appropriate, followed by Fisher post hoc test. \( P < 0.05 \) was considered statistically significant.
Results
Baseline Data of Dogs in CKD
As shown in Table 1, 1/2Nx and 5/6Nx reduced both GFR and RPF in a renal mass reduction–dependent manner. Although body weight was decreased in dogs with CKD (1/2Nx and 5/6Nx), neither mean arterial pressure nor heart rate was altered. Food consumption was not different among these groups. No significant difference in baseline CBF, LV end-diastolic pressure, or positive first derivative of LVP was noted among control, 1/2Nx, and 5/6Nx.

Blood glucose, insulin, and lipid levels were not different among the three groups (Table 2). Neither C-reactive protein nor the markers that are associated with endothelial dysfunction (fibrinogen, total plasminogen activator inhibitor-1, and von Willebrand factor) differed among these groups. Plasma homocysteine concentrations increased by reducing renal mass, although the differences did not attain statistical significance. Plasma ADMA concentrations were elevated in 1/2Nx and 5/6Nx, compared with control group. The l-arginine/ADMA ratio, a surrogate marker of NO production capacity, was significantly decreased in 1/2Nx and 5/6Nx. Plasma SDMA levels were elevated as renal mass was reduced. Of note, GFR was significantly correlated with plasma SDMA levels (r = −0.851, P < 0.0001), as well as serum creatinine (r = −0.749, P = 0.0013).

Effects of ACh and SNP on Coronary Circulation
The ACh-induced changes in CBF were markedly reduced in 1/2Nx and 5/6Nx, compared with control, 1/2Nx, and 5/6Nx. As shown in Table 1, 1/2Nx and 5/6Nx reduced both GFR and RPF in a renal mass reduction–dependent manner (28.3 ± 7.7, 14.6 ± 3.1, and 5.5 ± 2.3% increment, for control, 1/2Nx, and 5/6Nx, respectively; P < 0.05; each n = 5; Figure 2E). In contrast, no difference in arteriolar response to SNP was observed at any dosage examined among control, 1/2Nx, and 5/6Nx (each n = 5; Figure 2F). These results coincide with those of CBF in response to ACh and SNP.

Plasma NOx Levels and Expression Levels of eNOS and DDAH-I/II in Coronary Artery
Plasma NOx levels were decreased in both 1/2Nx and 5/6Nx as compared with those in control, although no significant difference was observed between 1/2Nx and 5/6Nx (control 22.1 ± 2.8 μmol/L [n = 5]; 1/2Nx 13.4 ± 0.9 μmol/L [n = 8]; P < 0.01; 5/6Nx 10.6 ± 2.4 μmol/L [n = 6]; P < 0.001; Figure 3A). The eNOS mRNA level in 1/2Nx did not differ from that in the control group, but marked downregulation was observed in coronary arteries from 5/6Nx (Figure 3B). Whereas no difference in DDAH-I mRNA expression levels was noted among these groups (n = 5; Figure 3C), the DDAH-II mRNA expression was pronouncedly downregulated in both 1/2Nx and 5/6Nx (n = 5; Figure 3D).

Figure 4 illustrates the immunohistochemical analysis on the coronary artery showing the protein expression levels of eNOS and DDAH-I/II. In the control group, eNOS, DDAH-I, and DDAH-II antibody produced intense labeling primarily along the endothelial layer (Figure 4). In the 1/2Nx and 5/6Nx groups, there was a loss of immunoreactivity of each molecule, the degree of which seemed to be greater in eNOS and DDAH-II than in DDAH-I.

Effect of atRA on CBF and Its Related Enzymes
Four-week treatment with atRA did not alter GFR (5/6Nx 17 [12 to 31] ml/min [n = 5]; 5/6Nx+atRA 22 [12 to 25] ml/min [n = 6]), BP (5/6Nx 105 [86 to 114] mmHg [n = 5]; 5/6Nx+atRA 102 [96 to 114] mmHg [n = 5]), or LV function.

Table 1. Baseline hemodynamic parameters in dogs with normal kidneys and CKD*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 5)</th>
<th>1/2Nx (n = 5)</th>
<th>5/6Nx (n = 5)</th>
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<tr>
<td>GFR (ml/min)</td>
<td>76 (54 to 85)</td>
<td>38 (29 to 47)</td>
<td>15 (12 to 46)</td>
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<tr>
<td>RPF (ml/min)</td>
<td>175 (147 to 296)</td>
<td>155 (144 to 179)</td>
<td>86 (61 to 113)</td>
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<tr>
<td>Body weight (kg)</td>
<td>18.0 (17.5 to 19.5)</td>
<td>17.0 (16.2 to 18.2)</td>
<td>16.7 (16.0 to 18.6)</td>
</tr>
<tr>
<td>MAP, conscious (mmHg)</td>
<td>105 (93 to 119)</td>
<td>100 (83 to 107)</td>
<td>111 (92 to 114)</td>
</tr>
<tr>
<td>HR, conscious (beats/min)</td>
<td>100 (82 to 115)</td>
<td>99 (87 to 106)</td>
<td>103 (93 to 122)</td>
</tr>
<tr>
<td>CBF (ml/min)</td>
<td>40 (29 to 65)</td>
<td>47 (32 to 56)</td>
<td>43 (27 to 58)</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>5.0 (3.9 to 8.4)</td>
<td>5.7 (4.1 to 6.7)</td>
<td>4.4 (4.1 to 5.3)</td>
</tr>
<tr>
<td>LVdP/dt (mmHg/s)</td>
<td>3230 (2674 to 4599)</td>
<td>2633 (1450 to 4023)</td>
<td>3996 (1785 to 4558)</td>
</tr>
</tbody>
</table>

*aData are median (range). 1/2Nx, heminephrectomy; 5/6Nx, five-sixths nephrectomy; CKD, chronic kidney disease; RPF, renal plasma flow; MAP, mean arterial pressure; HR, heart rate; CBF, coronary blood flow; LVEDP, left ventricular end-diastolic pressure; LVdP/dt, peak positive derivative of LV pressure.

*bP < 0.01, cP < 0.05 versus control.

dP < 0.05 versus 1/2Nx.
Table 2. Baseline characteristics in dogs with normal kidneys and CKD*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 5)</th>
<th>1/2Nx (n = 5)</th>
<th>5/6Nx (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>4.1 (3.6 to 5.2)</td>
<td>3.9 (3.2 to 4.2)</td>
<td>4.4 (3.9 to 4.8)</td>
</tr>
<tr>
<td>IRI (µU/ml)</td>
<td>5.0 (5.0 to 12.0)</td>
<td>4.7 (1.3 to 23.0)</td>
<td>5.0 (4.0 to 17.0)</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.29 (0.23 to 0.47)</td>
<td>0.34 (0.28 to 0.67)</td>
<td>0.36 (0.16 to 0.58)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.85 (3.03 to 4.50)</td>
<td>3.96 (3.67 to 4.91)</td>
<td>4.37 (3.78 to 4.73)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>2.87 (2.35 to 3.28)</td>
<td>2.90 (2.48 to 3.21)</td>
<td>3.39 (2.33 to 3.49)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>0.10 (0.03 to 0.13)</td>
<td>0.21 (0.08 to 0.52)</td>
<td>0.13 (0.05 to 0.28)</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>0.1 (0.0 to 0.1)</td>
<td>0 (0.0 to 0.1)</td>
<td>0 (0.0 to 0.1)</td>
</tr>
<tr>
<td>Fibrinogen (µmol/L)</td>
<td>8.0 (6.7 to 10.7)</td>
<td>10.4 (9.6 to 14.4)</td>
<td>7.7 (5.5 to 12.9)</td>
</tr>
<tr>
<td>Total PAI-1 (ng/ml)</td>
<td>10 (9 to 11)</td>
<td>10 (10 to 11)</td>
<td>10 (9 to 13)</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>6.3 (4.5 to 9.2)</td>
<td>7.7 (4.7 to 10.4)</td>
<td>7.8 (6.8 to 12.2)</td>
</tr>
<tr>
<td>L-arginine (µmol/L)</td>
<td>127.3 (90.6 to 144.0)</td>
<td>90.4 (81.0 to 121.7)</td>
<td>106.7 (97.4 to 112.9)</td>
</tr>
<tr>
<td>ADMA (µmol/L)</td>
<td>1.88 (1.68 to 2.54)</td>
<td>2.51 (2.11 to 3.55)b</td>
<td>3.84 (2.16 to 3.95)c</td>
</tr>
<tr>
<td>L-arginine/ADMA</td>
<td>56.5 (54.6 to 63.4)</td>
<td>33.7 (22.9 to 57.7)b</td>
<td>28.2 (25.4 to 51.7)c</td>
</tr>
<tr>
<td>SDMA (µmol/L)</td>
<td>0.29 (0.22 to 0.36)</td>
<td>0.49 (0.47 to 0.54)c</td>
<td>0.81 (0.49 to 0.85)de</td>
</tr>
</tbody>
</table>

*Data are median (range). ADMA, asymmetric dimethylarginine; IRI, immunoreactive insulin; PAI-1, plasminogen activator inhibitor-1; SDMA, symmetric dimethylarginine.

bP < 0.05, cP < 0.01, dp < 0.001 versus controls.

aP < 0.01 versus 1/2Nx.

Figure 1. Effects of acetylcholine (ACh) or sodium nitroprusside (SNP) on coronary blood flow (CBF) in chronic kidney disease (CKD) and correlation between plasma asymmetric dimethylarginine (ADMA) levels and endothelium-dependent vasodilation. (A) ACh-induced increase in CBF was markedly impaired in dogs with heminephrectomy (1/2Nx; □) and five-sixths nephrectomy (5/6Nx; △) compared with that in control dogs (○). (B) SNP-induced increase in CBF did not differ among the three groups. Percentage change from basal levels of CBF is expressed as mean ± SEM. **P < 0.01, ***P < 0.001 versus control; **P < 0.01 versus baseline.

(data not shown). Similarly, atRA had no effect on plasma homocysteine or ADMA levels. Treatment with atRA, however, markedly restored the ACh-induced changes in CBF in 5/6Nx (Figure 5A) without affecting the SNP-induced vasodilation (Figure 5B).

Whether the ameliorated vasodilator response to ACh was mediated by the alterations in eNOS or DDAH was examined (Figure 6). atRA increased plasma NOx levels but had no effect on plasma ADMA levels in 5/6Nx. Concomitantly, atRA restored the suppressed eNOS expression in 5/6Nx to the level of normal control (control n = 5; 5/6Nx n = 6; 5/6Nx+atRA n = 7; Figure 6C). In contrast, atRA failed to alter the expression of DDAH-I (Figure 6D) or DDAH-II (Figure 6E). In immunohistochemical analysis, the diminished eNOS staining was restored by the 4-wk treatment with atRA (Figure 7A), although atRA had no effect on the staining of DDAH-I (Figure 7B) or DDAH-II (Figure 7C).

Discussion

In this study, we demonstrated that the coronary artery manifests blunted responsiveness to ACh at the stage of mild to moderate CKD (Figure 1). In contrast, the vasodilator response to SNP is preserved even in moderate CKD. These observations indicate that the endothelium-dependent vasodilation of the coronary artery is impaired not only in advanced (i.e., moderate) CKD but also in early-stage (i.e., mild) CKD. Several recent studies have shown impaired endothelial function in various vascular beds, including isolated mesenteric (25), carotid (26), and subcutaneous resistance arteries (27). Our findings therefore provide direct in vivo evidence for endothelial dysfunction of the coronary artery. Of note, our experimental model did not manifest elevation in BP (Table 1), a finding that is in accordance with previous studies that showed that dogs with remnant kidneys are resistant to the induction of hypertension (28,29). Furthermore, cardiac dysfunction is not apparent in our model. It is most likely that impaired renal function per se constitutes a critical determinant of the coronary endothelial function in CKD, which may be associated with increased risks for cardiovascular events in CKD.

A growing body of evidence suggests that ADMA is responsible for the endothelial dysfunction in various disorders. Previous studies demonstrated the relationship between plasma ADMA levels and the degree of endothelial dysfunction of
forearm vascular beds in patients who were undergoing maintenance hemodialysis (30) and in hypertensive patients (31). Furthermore, coronary endothelial dysfunction is independently associated with the plasma ADMA concentration in men with early coronary atherosclerosis (32). Although reduced renal function is associated with attenuated coronary vasodilator capacity in patients with nonobstructive coronary artery disease (33), no investigation has examined the endothelial function of the coronary artery in CKD. We demonstrate that coronary endothelial dysfunction is already apparent in mild to moderate CKD, which endorses the recent findings that the risk for cardiovascular events is already high in patients with kidney disease and microalbuminuria (4). In this regard, CKD is characterized by hyperhomocysteinemia in humans (34). Because our study failed to show a significant elevation in homocysteine levels, our canine model may not completely reflect the situation in humans with CKD. In addition, a potential limitation of using dogs as an experimental animal is the low number in each study group.

Although our study indicates significant increases in systemic ADMA levels in CKD, the regulatory mechanisms of ADMA have not been elucidated. Because the kidney provides one route for clearance of ADMA, it is not surprising that plasma ADMA levels are elevated in patients with CKD (35). However, we have demonstrated that renal function is not a determinant of the plasma ADMA level because the ADMA level is not correlated with GFR, a finding that is in accordance with previous reports (36). Furthermore, induction of total nephrectomy was recently demonstrated to decrease, rather than increase, plasma ADMA levels in rats (37). Alternatively, our study demonstrated that the mRNA level of DDAH-II, a degrading enzyme for ADMA, is decreased in the tissues of the coronary artery at the early stage of CKD (Figure 3D). In this regard, we previously reported that the renal mRNA level of DDAH-II was decreased and suggested that this alteration was responsible for the endothelial dysfunction of renal arterioles (38). Therefore, the downregulation of DDAH-II in various

Figure 2. Effects of ACh or SNP on coronary arteriole diameters in CKD. (A through D) Representative images of epicardial coronary arterioles are shown. (A) Baseline in control dogs. (B) ACh 0.1 μg/kg per min in control dogs. (C) Baseline in 5/6Nx dogs. (D) ACh 0.1 μg/kg per min in 5/6Nx dogs. Diameters of arterioles were measured by marking the vessels on the camera screen. (E) ACh-induced coronary vasodilation was impaired in dogs with 1/2Nx (□) and 5/6Nx (△) compared with that in control dogs (○). (F) SNP elicited the coronary vasodilation, similar in magnitude in control, 1/2Nx, and 5/6Nx dogs. Percentage dilation from basal levels of diameter of coronary arteriole is expressed as mean ± SEM.*P < 0.05, **P < 0.01 versus control.

Figure 3. Plasma nitrite/nitrate (NOx) levels and mRNA expression of endothelial nitric oxide synthase (eNOS), dimethylarginine dimethylaminohydrolase-I (DDAH-I), and DDAH-II in the coronary artery of CKD. (A) Plasma NOx levels were decreased as renal function was impaired. The mRNA expressions of eNOS (B; control n = 5; 1/2Nx n = 8; 5/6Nx n = 6), DDAH-I (C; control n = 6; 1/2Nx n = 6; 5/6Nx n = 7), and DDAH-II (D; control n = 5; 1/2Nx n = 6; 5/6Nx n = 6) in the coronary artery were analyzed by real-time PCR. The mRNA expression levels of each molecule were normalized by that of 18S rRNA. The mRNA expression of eNOS and DDAH-II was markedly downregulated in the 1/2Nx and 5/6Nx groups. The expression of DDAH-I was also reduced, but the change did not attain statistical significance. Results are expressed as means ± SEM. *P < 0.01, ***P < 0.001 versus control; †P < 0.05 versus 1/2Nx.
tissues, including the kidney and coronary vessels, would contribute to the elevation of circulating ADMA levels. It was demonstrated that several factors (e.g., oxidized LDL [39], homocysteine [40], hyperglycemia [41]) reduce the activity of DDAH without the alteration of its expression. Furthermore, oxidative stress downregulates the expressions of DDAH-II (42). Whereas we show distinct regulation of DDAH-I and DDAH-II in dogs with CKD (Figure 3), Matsuguma et al. (43) demonstrated the downregulation of both DDAH-I and DDAH-II in subtotally nephrectomized rats, which manifest elevated BP. Although the reason for these divergent effects of CKD is unknown, different experimental settings such as animal species and BP might affect these differences.

Of interest, our study shows the intimate correlation between GFR (estimated by inulin clearance) and SDMA ($r = 0.851$). This intriguing finding is in accordance with the results of a recent meta-analysis (44) and therefore lends support to the premise that SDMA constitutes a novel marker of renal function. Further investigations would establish the role of SDMA as a reliable index of renal function.

Besides ADMA as an endogenous substance for coronary endothelial dysfunction, our study has demonstrated that the mRNA expression of eNOS is downregulated in coronary arterial tissues in moderate (i.e., 5/6Nx) CKD (Figure 3B). Furthermore, our findings that atRA ameliorates endothelium-

mocysteine [40], hyperglycemia [41]) reduce the activity of DDAH without the alteration of its expression. Furthermore, oxidative stress downregulates the expressions of DDAH-II (42). Whereas we show distinct regulation of DDAH-I and DDAH-II in dogs with CKD (Figure 3), Matsuguma et al. (43) demonstrated the downregulation of both DDAH-I and DDAH-II in subtotally nephrectomized rats, which manifest elevated BP. Although the reason for these divergent effects of CKD is unknown, different experimental settings such as animal species and BP might affect these differences.

Of interest, our study shows the intimate correlation between GFR (estimated by inulin clearance) and SDMA ($r = -0.851$). This intriguing finding is in accordance with the results of a recent meta-analysis (44) and therefore lends support to the premise that SDMA constitutes a novel marker of renal function. Further investigations would establish the role of SDMA as a reliable index of renal function.

Besides ADMA as an endogenous substance for coronary endothelial dysfunction, our study has demonstrated that the mRNA expression of eNOS is downregulated in coronary arterial tissues in moderate (i.e., 5/6Nx) CKD (Figure 3B). Furthermore, our findings that atRA ameliorates endothelium-
dependent vasodilation (Figure 5) and upregulates eNOS expression (Figure 6C) suggest that the suppressed eNOS activity accounts for the impaired endothelium-dependent vasodilation of the coronary artery at least in part in moderate CKD. The downregulation of eNOS in CKD was previously reported in the aortic tissue (10) and gastric mucosa (11) of rat model with five-sixths nephrectomy, and parathyroid hormone and the change in intracellular calcium concentration were proposed as a potential mechanism (10). Alternatively, CKD and its metabolic consequence, including increased oxidative stress, are supposed to play substantial roles in eNOS downregulation (45). Paradoxic, previous studies showed elevated plasma NOx levels in patients with CKD, which was probably due to decreased excretion of NOx (46,47). To the extent that pulsatile stretch and distension of the arterial wall favor NO release in hypertension (48), it is speculated that lack of hypertension in our canine model and the subsequent reduction in systemic NO production may result in no increases in plasma NOx levels. Of note, in mild (i.e., 1/2Nx) CKD, the eNOS expression was relatively preserved, whereas the DDAH-II expression was markedly downregulated (Figure 3). These findings suggest that different mechanisms affect the regulation of eNOS and DDAH-II expression, probably because these molecules are transcribed from different genes and regulated by different promoters of each gene.

Therapeutic strategy for cardiovascular protection in CKD merits comment. We recently demonstrated that the treatment with a peroxisome proliferator–activated receptor-γ ligand, pioglitazone, enhances renal DDAH-II expression and urinary NOx excretion, as well as reduces plasma ADMA levels, in hypertensive rat models (49). Furthermore, atRA is reported to increase DDAH-II expression and reduce ADMA in cultured endothelial cells without affecting eNOS expression (22). Although our study failed to show upregulation of DDAH-II by the 4-wk treatment with atRA, it markedly upregulates the eNOS expression and reverses the endothelium-dependent vasodilation of the coronary artery in vivo (Figures 6 and 7). These observations suggest that the in vivo effects of atRA on eNOS and DDAH-II expression in CKD differ from those in the in vitro condition. Nevertheless, it is tempting to speculate that these agents alter the course of CKD by manipulating NO and ADMA levels and ameliorating endothelial dysfunction. Of course, further investigations are required to clarify this important issue.

Conclusion
This study demonstrates impaired coronary endothelial function in dogs with CKD, which is apparent at the mild to moderate stage of CKD. The derangement is presumably attributed to ADMA-mediated NO suppression and/or the decreased expression of eNOS. These data underscore CKD as a potential cardiovascular risk factor and warrant early therapeutic intervention in CKD for the prevention of cardiovascular events.

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Disclosures
None.

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