Original Article

Role of Asymmetrical Dimethylarginine in Renal Microvascular Endothelial Dysfunction in Chronic Renal Failure with Hypertension

Ken OKUBO, Koichi HAYASHI, Shu WAKINO, Hiroto MATSUDA,
Eiji KUBOTA, Masanori HONDA, Hirobumi TOKUYAMA,
Tokunori YAMAMOTO*, Fumihiko KAJIYA**, and Takao SARUTA

We examined whether endothelial function of the renal microcirculation was impaired in a model of chronic renal failure (CRF), and further assessed the role of asymmetrical dimethylarginine (ADMA) and its degrading enzyme, dimethylarginine dimethylaminohydrolase (DDAH), in mediating the deranged nitric oxide (NO) synthesis in CRF. CRF was established in male mongrel dogs by subtotal nephrectomy, and the animals were used in experiments after a period of 4 weeks. The endothelial function of the renal afferent and efferent arterioles was evaluated according to the response to acetylcholine, using an intravital needle-lens charge-coupled device camera. Intrarenal arterial infusion of acetylcholine ($0.01 \mu\text{g/kg/min}$) elicited $22 \pm 2\%$ and $20 \pm 2\%$ dilation of the afferent and efferent arterioles in normal dogs. In dogs with CRF, this vasodilation was attenuated (afferent, $12 \pm 2\%$; efferent, $11 \pm 1\%$), and the attenuation paralleled the diminished increments in urinary nitrite+nitrate excretion. In the animals with CRF, plasma concentrations of homocysteine (12.2 ± 0.7 vs. $6.8 \pm 0.4 \mu\text{mol/l}$) and ADMA were elevated (2.60 ± 0.13 vs. $1.50 \pm 0.08 \mu\text{mol/l}$). The inhibition of S-adenosylmethionine-dependent protein arginine N-methyltransferase by adenosine dialdehyde decreased plasma ADMA levels, and improved the acetylcholine-induced changes in urinary nitrite+nitrate excretion and arteriolar vasodilation. Acute methionine loading impaired the acetylcholine-induced renal arteriolar vasodilation in CRF, but not normal dogs, and the impairment in CRF dogs coincided with the changes in plasma ADMA levels. Real-time polymerase chain reaction revealed downregulation of the mRNA expression of DDAH-II in the dogs with CRF. Collectively, these results provide direct *in vivo* evidence of endothelial dysfunction in canine CRF kidneys. The endothelial dysfunction was attributed to the inhibition of the NO production by elevated ADMA, which involved the downregulation of DDAH-II. The deranged NO metabolic pathway including ADMA and DDAH is a novel mechanism for the aggravation of renal function. (*Hypertens Res* 2005; 28: 181–189)

Key Words: asymmetrical dimethylarginine, renal disease, nitric oxide, renal microcirculation, endothelium

Introduction

Cardiovascular disease is a major cause of morbidity and

mortality in patients with chronic renal failure (CRF) (1, 2), and renal disease *per se* increases the incidence of cardiovascular events even in the presence of macro- (3) or microalbuminuria (4). Various factors have been identified as risk

From the Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan; *Department of Urology, Nagoya University Graduate School of Medicine, Nagoya, Japan; and **Department of Cardiovascular Physiology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan.

Address for Reprints: Koichi Hayashi, M.D., Ph.D., Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160–8582, Japan. E-mail: khayashi@sc.itc.keio.ac.jp

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Table 1. Baseline Hemodynamic and Blood Parameters in Dogs with Normal Kidneys and Chronic Renal Failure

	Control	CRF
<i>n</i>	7	7
Body weight (kg)	12.2±1.1	10.4±1.0*
MAP (mmHg) (conscious)	96±3	119±2**
HR (beats/min) (conscious)	114±4	95±8*
Hematocrit (%)	49±2	39±2**
Serum creatinine (μmol/l)	64.6±2.7	188.5±26.5**
Total cholesterol (mmol/l)	3.25±0.05	3.35±0.25
HDL-cholesterol (mmol/l)	2.69±0.11	2.77±0.12
Triglyceride (mmol/l)	0.21±0.03	0.38±0.05*
Creatinine clearance (ml/min)	31.3±1.5	13.2±0.8**

CRF, chronic renal failure; MAP, mean arterial pressure; HR, heart rate; HDL, high density lipoprotein. Data are expressed as the mean±SEM. * $p<0.05$ vs. controls. ** $p<0.01$ vs. controls.

factors for cardiovascular events in CRF, including a modest increase in blood pressure (5, 6), left ventricular hypertrophy (7), and elevated lipoprotein (a) (8). Recently, hyperhomocysteinemia has been recognized as an independent risk factor promoting atherosclerosis by inducing endothelial dysfunction (9), and thus constitutes a potential cardiovascular risk factor in CRF (10). Although the mechanism for hyperhomocysteinemia-induced cardiovascular disease still remains controversial, several lines of studies suggest that homocysteine limits the bioavailability of NO (11) and increases oxidative stress (12), thereby favoring hyper-reactivity of vascular beds (13). It is surmised therefore that in CRF, endothelial dysfunction within the renal microvasculature accelerates the renal injury, which would constitute a vicious circle for the progression of end-stage CRF. Nevertheless, the role of homocysteine and the disturbed NO production in mediating the development of renal microvascular dysfunction has not been elucidated.

A growing body of evidence has accrued that CRF is associated with deranged NO metabolism. Valance *et al.* (14) originally demonstrated the presence of an endogenous NO synthase inhibitor, asymmetric dimethylarginine (ADMA), and subsequent studies have shown that accumulation of ADMA is an important risk factor for cardiovascular disease in CRF (15, 16). ADMA is biosynthesized from arginine by *S*-adenosylmethionine-dependent protein arginine *N*-methyltransferase (PRMT), which transmethyates *S*-adenosylmethionine, an intermediate in the conversion of methionine to homocysteine (17). Furthermore, ADMA degradation is regulated by dimethylarginine dimethylaminohydrolase (DDAH), which has recently been shown to have two isoforms, DDAH-I and DDAH-II, and exhibits different tissue distributions and regulations for each isoform (18, 19). Although both homocysteine and ADMA are suggested to be novel candidates causing endothelial dysfunction in CRF by impairing NO activity, the precise regulatory mechanisms for

Table 2. Baseline Characteristics of Dogs with Normal Kidneys and Chronic Renal Failure

	Control	CRF
<i>N</i>	7	7
von Willebrand factor (%)	11.3±0.8	31.0±8.0*
PAI-1 (nmol/l)	352±127	848±190 [#]
Homocysteine (μmol/l)	6.8±0.4	12.2±0.7**
ADMA (μmol/l)	1.50±0.08	2.60±0.13**

CRF, chronic renal failure; PAI-1, plasminogen activator inhibitor-1; ADMA, asymmetrical dimethylarginine. Data are expressed as the mean±SEM. * $p<0.05$ vs. controls. ** $p<0.01$ vs. controls. [#] $p=0.05$ vs. controls.

these enzymes have not been elucidated in CRF.

In the present study, we directly examined the endothelial function of the renal microvasculature in CRF by using an intravital needle-lens charge-coupled device (CCD) camera technique, which allowed direct visualization of the renal microvessels in an *in vivo*, *in situ*, and relatively intact experimental setting (20, 21). We observed that the acetylcholine (ACh)-induced vasodilation of the renal microcirculation and the NO utilization were impaired in CRF. Furthermore, we observed an elevation in plasma ADMA and suppressed expression of the renal DDAH-II mRNA level in CRF. The pharmacological inhibition of PRMT by adenosine dialdehyde restored the elevated plasma ADMA level and the blunted microvascular response to ACh. These findings suggest that the accumulation of ADMA by the suppressed DDAH-II expression within the kidney is one possible mechanism for endothelial dysfunction in CRF.

Methods

All experimental procedures were conducted according to the institutional guidelines of the Animal Care Committee. Fifty adult male mongrel dogs (9–14 kg) were fed a standard diet (Oriental Yeast Co., Tokyo, Japan) and were divided into an ACh protocol group (normal controls: $n=7$; CRF: $n=7$), an ACh+adenosine dialdehyde protocol group (normal controls: $n=7$; CRF: $n=7$), a methionine loading protocol group (normal controls: $n=6$; CRF: $n=6$), and a protocol group for the quantification of DDAH mRNA expression (normal controls: $n=5$; CRF: $n=5$). Dogs in the CRF groups were anesthetized with sodium pentobarbital (25 mg/kg), and CRF was induced by ligation of two or three branches of the left renal artery and contralateral nephrectomy. Dogs in the normal control groups were anesthetized with sodium pentobarbital (25 mg/kg) and sham-operated. Four weeks after the operation, dogs of all groups underwent the same protocol. Animal preparations were detailed in our previous publications (20–22). An electromagnetic flow probe was placed around the left renal artery for measurement of renal blood flow (RBF).

Renal microcirculation was assessed with an intravital nee-

Fig. 1. Visualization of renal microvessels and effects of acetylcholine on renal microvessels. The diameter of afferent arterioles (AFF) in chronic renal failure (CRF) dogs was greater than that in normal dogs, whereas no difference in efferent arteriole (EFF) diameter was noted (A, B). Furthermore, the acetylcholine-induced vasodilation of AFF and EFF was blunted in CRF dogs (triangles, $n=7$) compared with that in normal dogs (circles, $n=7$; C, D). The arteriolar responses to acetylcholine were expressed as a percentage of nitroprusside-induced vasodilation. Results are expressed as the mean $\pm$ SEM. SNP, sodium nitroprusside. $**p < 0.01$ vs. baseline; $^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$ vs. normal dogs. $^{\S}p < 0.05$ vs. 0.01 $\mu\text{g/kg/min}$ acetylcholine.

dle-lens CCD camera (VMS-1210; Nihon Kohden, Tokyo, Japan) (20, 21). After the surgical procedure and instrumentation, a CCD probe was introduced into the left kidney. The animals were allowed to equilibrate for 60 min before the initiation of experimental protocols.

Protocols

After allowing the basal hemodynamics to stabilize for 20 min, ACh (Sigma, St. Louis, USA) or sodium nitroprusside (SNP; Maruishi Seiyaku, Tokyo, Japan) was infused directly into the renal artery at increasing doses of 0.0001 to 0.01 $\mu\text{g/kg/min}$ for ACh, and 0.002 $\mu\text{g/kg/min}$ for SNP. The effects of these agents on RBF, urinary nitrite+nitrate (NOx) and the renal microvascular tone were evaluated. A 30-min interval after each ACh dose was required for stabilization of the response.

To examine whether the ADMA-mediated NO inhibition was responsible for the impaired renal endothelial response, the effects of adenosine dialdehyde, a PRMT inhibitor, on the plasma ADMA concentration and the ACh induced-response of renal microcirculation were evaluated. After the stabiliza-

tion of basal hemodynamics, ACh (0.01 $\mu\text{g/kg/min}$) was administered into the renal artery. Following the 30-min observation of the ACh-induced response, adenosine dialdehyde (15 $\mu\text{g/kg/min}$; Sigma) was infused into the renal artery and the effects of these agents were assessed.

For the final protocol, dogs of both the CRF and normal kidney groups received L-methionine (100 mg/kg; Ajinomoto, Tokyo, Japan) orally at 7:00 in the morning. A standard breakfast was served 2 h after methionine administration. Plasma homocysteine and ADMA concentrations were assayed at 0, 5, 8, 12, and 24 h after methionine administration. Likewise, urinary NOx excretion and renal microvascular responses to ACh (0.01 $\mu\text{g/kg/min}$) were evaluated at 0, 5, and 12 h after methionine administration.

Biochemical Analyses

Plasma homocysteine and ADMA were determined by high performance liquid chromatography (23, 24). Urinary NOx concentrations were evaluated using the Griess reaction (21, 25).

Fig. 2. Effects of acetylcholine on renal blood flow (RBF), and urinary nitrate/nitrite excretion. Acetylcholine-induced increases in RBF and urinary nitrate+nitrite (NOx) excretion were smaller in dogs with chronic renal failure (CRF; triangles, $n=7$) than in normal dogs (circles, $n=7$). Results are expressed as the mean $\pm$ SEM. SNP, sodium nitroprusside. * $p < 0.05$, ** $p < 0.01$ vs. baseline. † $p < 0.05$, †† $p < 0.01$ vs. normal dogs. ‡ $p < 0.05$ vs. 0.01 $\mu\text{g/kg/min}$ acetylcholine.

Fig. 3. Effects of adenosine dialdehyde on plasma asymmetrical dimethylarginine, homocysteine, urinary nitrate/nitrite excretion, and renal hemodynamics. Plasma asymmetrical dimethylarginine (ADMA) and homocysteine were elevated in dogs with chronic renal failure (CRF). Adenosine dialdehyde (AD; 15 $\mu\text{g/kg/min}$) suppressed plasma ADMA, but not homocysteine. In dogs with normal kidneys, AD had no effect on the changes in urinary nitrate+nitrite (NOx) excretion, renal blood flow (RBF), or renal arteriolar diameter induced by intrarenal infusion of acetylcholine (ACh; 0.01 $\mu\text{g/kg/min}$). In CRF dogs, the ACh-induced changes in these parameters were diminished, and were partially restored by the pretreatment with AD. Results are expressed as the mean $\pm$ SEM ($n=7$). * $p < 0.05$ vs. baseline. † $p < 0.05$, †† $p < 0.01$ vs. normal dogs.

mRNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the canine renal cortex with Trizol Reagent (Invitrogen, Carlsbad, USA). Total RNA (50 ng) was reverse-transcribed for cDNA synthesis with the SuperScript First-Strand Synthesis System (Invitrogen). For

the quantification of mRNA expression of DDAH-I/II, real-time PCR was performed using the ABI PRISM-7700 sequence detector (PE Applied Biosystems, Tokyo, Japan). SYBER Green I Dye (PE Applied Biosystems) was utilized for detection of the PCR reaction. Each set of primers yielded a single amplified PCR product with a sequence identical to one of those published in GenBank.

Fig. 4. Temporal profiles of the effects of oral methionine loading on plasma concentrations of homocysteine and asymmetrical dimethylarginine, urinary nitrate/nitrite excretion and blood pressure. Acute methionine loading evoked prominent increases in plasma homocysteine levels in dogs with normal and chronic renal failure (CRF) kidneys, whereas a marked elevation in plasma asymmetrical dimethylarginine (ADMA) level was seen in CRF dogs. Urinary nitrate+nitrite (NOx) excretion was decreased only in CRF dogs. In parallel, exaggerated elevations in blood pressure were observed in CRF dogs. Results are expressed as the mean $\pm$ SEM ($n=6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 0 h. † $p < 0.05$, †† $p < 0.01$ vs. normal dogs.

Statistics

Results are expressed as the mean $\pm$ SEM. Data were analyzed by 1-way/2-way ANOVA, as appropriate, followed by Bonferroni's post hoc test (StatView, SAS Institute Inc., Cary, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Baseline Characteristics of CRF Dogs

Table 1 shows baseline characteristics of the dogs with CRF and the normal controls. After 4 weeks of five-sixths nephrectomy, body weight, heart rate, and glomerular filtration rate were decreased. Mean arterial pressure did not change at 2 weeks of subtotal nephrectomy (104 ± 6 mmHg, $p > 0.1$, $n=7$), but a significant elevation was observed at 4 weeks. Serum creatinine levels were elevated from 64.6 ± 2.7 to 188.5 ± 26.5 $\mu\text{mol/l}$. Although triglyceride levels were elevated, total cholesterol and high density lipoprotein (HDL)-cholesterol levels did not change. Plasma levels of von Willebrand factor and plasminogen activator inhibitor-1 (PAI-1), markers for endothelial function, were elevated in the dogs with CRF (Table 2). Both plasma homocysteine and ADMA concentrations were increased. Plasma ADMA concentrations were elevated at 2 weeks of five-sixths nephrectomy (2.20 ± 0.14 $\mu\text{mol/l}$, $p < 0.01$, $n=7$).

Effect of ACh and SNP on Renal Microcirculation

Using an intravital CCD camera, we examined whether CRF altered the endothelial function of renal afferent and efferent arterioles *in vivo*. In normal dogs, the basal diameters of the afferent and efferent arterioles were 14.4 ± 0.2 ($n=7$) and 12.5 ± 0.5 μm ($n=7$), respectively (Fig. 1A). Direct intrarenal infusion of ACh caused dose-dependent dilation of the afferent and efferent arterioles, with $22 \pm 2\%$ and $20 \pm 2\%$ increments in diameter observed at 0.01 $\mu\text{g/kg/min}$, respectively. Further administration of SNP had no effect on either afferent or efferent arterioles.

In CRF dogs, the basal afferent arteriolar diameter was greater (24.7 ± 2.2 μm , $n=7$) than that in normal controls ($p < 0.01$; Fig. 1B), but no difference in efferent arteriolar diameter was noted between CRF (13.0 ± 0.8 μm , $n=7$) and normal dogs ($p > 0.5$). Furthermore, the vasodilator response to ACh was diminished in CRF dogs; at 0.01 $\mu\text{g/kg/min}$, only $12 \pm 2\%$ and $11 \pm 1\%$ increments were observed in the afferent and efferent arterioles, respectively. The subsequent addition of SNP dilated both arterioles by an increment of $16 \pm 2\%$ and $18 \pm 2\%$ of the baseline value, respectively. When the arteriolar responses to ACh were expressed as a percentage of the SNP-induced vasodilation, it was evident that the responses in CRF were blunted compared with those in normal dogs (Fig. 1C and D). The vasodilator response to ACh (0.01 $\mu\text{g/kg/min}$) was also diminished at 2 weeks of subtotal nephrectomy (afferent, $15 \pm 1\%$ increments, $p = 0.001$ vs. normal controls; efferent, $14 \pm 4\%$ increments, $p = 0.03$ vs. normal

Fig. 5. Sequential changes in acetylcholine-induced actions on renal arterioles and urinary nitrate/nitrite excretion during oral methionine loading. Oral methionine loading had no effect on the arteriolar responses to acetylcholine (ACh) or urinary nitrate+nitrite (NOx) excretion in normal dogs. In contrast, in dogs with chronic renal failure (CRF), the ACh-induced dilation of the renal arterioles and the increase in urinary NOx excretion were markedly attenuated at 5 h of acute methionine loading, and these attenuations tended to persist at 12 h. Results are expressed as the mean $\pm$ SEM ($n=6$).

controls).

We further investigated the effects of ACh on RBF and urinary NOx excretion in CRF (Fig. 2). Baseline levels of RBF (61 ± 6 ml/min, $n=7$) and urinary NOx excretion (11.0 ± 2.4 nmol/min, $n=7$) in the animals with CRF were less than those in normal controls (RBF, 185 ± 11 ml/min, $n=7$, $p<0.01$; urinary NOx, 32.2 ± 11.0 nmol/min, $n=7$, $p<0.01$). In normal dogs, RBF and urinary NOx excretion were augmented in a dose-dependent manner; at $0.01 \mu\text{g/kg/min}$, ACh elicited $28 \pm 3\%$ increments in RBF and $239 \pm 56\%$ increments in urinary NOx excretion. In CRF dogs, blunted ACh-induced increases in RBF and urinary NOx excretion were observed.

Effects of PRMT Inhibition on Renal Hemodynamics and Microcirculation

In normal dogs, the administration of adenosine dialdehyde had no effect on plasma ADMA/homocysteine levels (Fig. 3), urinary NOx excretion, RBF or renal arteriolar diameters ($n=7$). Furthermore, adenosine dialdehyde did not alter the ACh-induced changes in these parameters ($n=7$, Fig. 3). In CRF dogs, however, adenosine dialdehyde caused a significant decrease in plasma ADMA concentration (from 2.58 ± 0.11 to $2.23 \pm 0.10 \mu\text{mol/l}$, $n=7$, $p<0.05$), and increases in urinary NOx excretion (from 25.0 ± 6.2 to 61.0 ± 7.1 nmol/min, $n=7$, $p<0.05$), RBF (from 66 ± 2 to 76 ± 2 ml/min, $p<0.05$) and renal arteriolar diameters (afferent, from 26.4 ± 0.6 to $28.6 \pm 0.5 \mu\text{m}$, $p<0.05$; efferent, from 15.0 ± 0.2 to $16.5 \pm 0.3 \mu\text{m}$, $p<0.05$). Plasma homocysteine levels tended to decrease, although the changes did not attain statistical significance.

In CRF dogs, the diminished ACh ($0.01 \mu\text{g/kg/min}$)-induced increases in urinary NOx excretion (from 11.0 ± 2.4 to 25.0 ± 6.2 nmol/min, $p<0.05$, $n=7$) was prominently restored by adenosine dialdehyde (to 61.0 ± 7.1 nmol/min) (Fig. 3). Similarly, the ACh-induced changes in RBF and the

diameters of afferent and efferent arterioles were enhanced by adenosine dialdehyde.

Effects of Oral Methionine Loading on Plasma Homocysteine and ADMA Concentrations

Acute oral methionine loading markedly elevated the plasma homocysteine levels in both normal and CRF dogs (Fig. 4). In contrast, the methionine administration caused only a modest elevation in the plasma ADMA concentration in normal dogs, whereas a prominent increase in the plasma ADMA concentration was noted in CRF dogs. Similarly, methionine loading had only a modest, nonsignificant effect on urinary NOx excretion in normal dogs, but a significant decrease was observed 5 h after methionine loading in CRF dogs. Concomitantly, blood pressure was elevated in both normal and CRF dogs, although the changes were greater in CRF dogs.

In parallel with the changes in plasma ADMA concentrations, the ACh ($0.01 \mu\text{g/kg/min}$)-induced vasodilation of renal afferent and efferent arterioles was markedly impaired in CRF dogs at 5 h of oral methionine loading, and this tendency persisted at 12 h ($p=0.1$ vs. 0 h; Fig. 5). In contrast, in normal dogs, methionine had no effect on the ACh-induced vasodilation of these arterioles. These results paralleled the changes in the urinary NOx excretion induced by ACh; the ACh-induced increments in urinary NOx excretion were markedly attenuated at 5 h of methionine loading in CRF dogs, whereas only a modest inhibitory tendency was seen in normal dogs.

Expression of DDAH-I/II in Normal and Remnant Kidneys

The mRNA expression level of each isoform of DDAH in the kidney was assessed by real-time PCR. The mRNA levels of DDAH-I tended to be downregulated in CRF, although the

Fig. 6. mRNA expression of dimethylarginine dimethylaminohydrolase (DDAH) in the kidney. The mRNA expression of DDAH-I and DDAH-II in the normal kidneys and the remnant kidneys with chronic renal failure (CRF) was analyzed by real-time polymerase chain reaction. The ratio between the mRNA levels of each isoform of DDAH and the mRNA level of GAPDH, a constitutively expressed isoform, was used for the comparison. The sequences for the forward and reverse primers were: 5'-catggctggcctaactaat-3' and 5'-tgagttgtcatagcgggtggtc-3' for DDAH-I, 5'-aggtaccagggtgacatcagaga-3' and 5'-cagctgctgactgcctcttc-3' for DDAH-II, and 5'-ggacaaatcaacgaggtgct-3' and 5'-ggacaaatcaacgaggtgct-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. The amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Results are expressed as the mean $\pm$ SEM ($n=5$). n.s., not significant.

difference did not attain statistical significance (Fig. 6). In contrast, the mRNA levels of DDAH-II were markedly down-regulated in the dogs with CRF ($p<0.01$ vs. normal controls, $n=5$).

Discussion

In the present study, we have demonstrated that the endothelium-dependent vasodilation of renal microvessels is impaired in subtotal nephrectomized CRF dogs, with the use of a novel technique that directly visualizes the *in vivo*, *in situ*, and relatively intact renal microcirculation, *i.e.*, an intravital needle-lens CCD camera technique (20, 21). The alteration in the endothelium-dependent vasodilation of renal arterioles is closely linked to the plasma ADMA and NO production, rather than the homocysteine concentration. Finally, the modification of the plasma ADMA level by the blockade of PRMT activity or methionine loading alters the endothelial function in CRF, which parallels the changes in urinary NO_x excretion. Collectively, these observations strongly suggest an important contribution of ADMA, an endogenous NO synthesis inhibitor, to the renal microvascular abnormality in CRF, which would account in part for the deranged renal and/or systemic NO production in CRF. Although endothelial

dysfunction in CRF has been documented in various experimental settings (26, 27), and indeed is anticipated from the increased plasma von Willebrand factor and PAI-1 (Table 2), no investigations have been conducted examining the endothelial function at the renal microvascular level *in vivo*. The present study thus offers the first direct evidence for the impaired endothelial function of the renal microvasculature in CRF.

It is worth noting that the afferent arterioles in CRF dogs have larger diameters than those in normal dogs. Although the mechanism of the afferent arteriolar dilation in CRF remains unestablished, the diminished tone of this vessel would promote glomerular hypertension and the subsequent development of renal injury. Furthermore, an increase in the diameters of the afferent arterioles of CRF dogs may augment the wall tension of these arterioles, and thereby facilitate endothelial dysfunction. Nevertheless, the diminished response of the efferent arterioles to ACh clearly indicates a CRF-induced alteration in the responsiveness of this vessel segment.

Among various factors responsible for the endothelial dysfunction in CRF, we have focused on two substances that are reported to contribute to the regulation of vascular tone in various vascular beds, ADMA and homocysteine. ADMA has been shown to inhibit NO synthase by competing with L-arginine (28), and plasma ADMA levels are elevated in both patients (14, 16) and experimental animals with CRF (Table 2). It has been demonstrated that elevated plasma ADMA concentrations are significantly related to the degree of impaired endothelium-dependent forearm vasodilation (29). Indeed, the present study showed that the reduction in the plasma ADMA level by adenosine dialdehyde (17) enhanced the urinary NO_x excretion and restored the ACh-induced changes in renal hemodynamic parameters, including RBF and afferent and efferent arteriolar diameters in dogs with CRF, but not in those with normal renal function (Fig. 3). Conversely, acute ADMA elevation by methionine loading was associated with elevated blood pressure (Fig. 4) and a blunted vasodilator response to ACh in CRF dogs (Fig. 5). ADMA therefore constitutes an important factor contributing to the derangement in endothelial function in renal microcirculation, and may also be responsible for the acceleration of cardiovascular events in CRF (16). In this regard, we also found elevated plasma ADMA concentrations (2.20 ± 0.14 μ mol/l) and the diminished vasodilator response to ACh at 2 weeks of five-sixths nephrectomy, at which stage mean arterial pressure was unaltered (104 ± 6 mmHg). In concert, it is conjectured that elevated plasma ADMA concentrations and endothelial dysfunction are attributable to the changes in internal milieu induced by CRF, although the elevated blood pressure *per se* may also modify ADMA production at advanced stages of CRF. It is of note that the plasma triglyceride level is greater in the dogs with CRF than in the normal controls in the present study, and acute hypertriglyceridemia following high fat diet intake has been reported to be associ-

ated with endothelial impairment (30). Nevertheless, it has also been demonstrated that chronic hypertriglyceridemia does not affect endothelial function (31). Further studies are required to clarify this issue.

The present study also demonstrated an increased level of plasma homocysteine in CRF dogs. Previous studies have shown that hyperhomocysteinemia is associated with impaired endothelial function in animals and humans (12, 28). Although the precise mechanisms of the impairment are not fully determined, a direct NO-quenching effect by oxidative stress is one possible mechanism (12). Recently, Böger *et al.* (32) reported that acute hyperhomocysteinemia induced by methionine administration increases plasma ADMA concentrations, and impairs the flow-mediated vasodilation of the brachial artery in healthy human subjects. They suggested that the impaired vasodilation during the acute homocysteine elevation may be attributed to an increase in ADMA. In the present study, we examined the effect of acute methionine loading on plasma homocysteine and ADMA levels and simultaneously assessed the ACh-induced vasodilation of renal arterioles. Our results showed that, although nearly the same level of plasma homocysteine was achieved in normal and CRF dogs during the methionine administration, the impairment in the ACh-induced renal arteriolar vasodilation by methionine was associated with the level of ADMA, but not homocysteine (Figs. 4 and 5). Furthermore, the administration of adenosine dialdehyde failed to alter plasma homocysteine levels, but decreased plasma ADMA and ameliorated the renal hemodynamic responses to ACh (Fig. 3). Taken together, these observations favor the premise that ADMA plays a substantial role in mediating the impaired endothelial function in CRF.

Although the present study clearly indicates that ADMA plays an important role in the endothelial dysfunction in CRF, the regulatory mechanism of the plasma ADMA level has not been fully explored. It is reasonable to speculate that the elevated ADMA concentration is attributable to inadequate renal clearance (33). Alternatively, as demonstrated in the present study, the metabolism of ADMA may be altered in CRF. Thus, the mRNA of DDAH-II, an ADMA-degrading enzyme, is downregulated in kidneys from CRF dogs (Fig. 6), whereas in our preliminary experiments no change was observed in the level of PRMT, an ADMA-producing enzyme. Immunohistochemical analysis has shown that DDAH is widely distributed within the rat kidney, including the endothelium in the glomeruli and renal vasculature as well as the proximal tubules and macula densa (34). Recently, Leiper *et al.* (18) cloned two isoforms of human DDAH, and found that DDAH-I predominates in tissues expressing neuronal NOS, whereas DDAH-II is distributed in the regions where endothelial NOS is co-localized. Therefore, decreased expression of DDAH-II in the renal vasculature of the remnant kidney might result in the accumulation of ADMA, and this would augment the inhibitory actions of ADMA on endothelial NOS in renal microvessels. Furthermore, an alteration at the level

of mRNA is consistent with the change in the enzyme activity; acute methionine loading elicited a marked elevation in ADMA concentration in CRF, whereas in normal controls only a modest increase in the plasma ADMA level was noted (Fig. 4). Taken together, our studies indicate that the downregulation of DDAH-II constitutes a critical determinant of the CRF-induced impairment of renal microcirculation.

In this regard, Stühlinger *et al.* (35) have recently demonstrated that homocysteine impairs the NO synthase pathway through the inhibition of DDAH in cultured endothelial cells. Whereas their findings observed *in vitro* show an inhibitory action of homocysteine on DDAH activity and subsequently a positive correlation between homocysteine and ADMA levels, our observations obtained in normal dogs indicate dissociation between plasma homocysteine and ADMA levels. These discrepant findings may be attributed to the different experimental settings (*e.g.*, *in vivo* vs. *in vitro*). Furthermore, it is plausible that additional mechanisms may contribute to the suppressed DDAH activity and expression in CRF. Intriguingly, our results indicate that each DDAH isoform is differently regulated in the kidney, a finding consistent with the previous report in pulmonary hypertension (19). Further studies will be needed to elucidate the regulatory mechanism of DDAH-II in CRF.

In conclusion, the present study provides direct evidence of the endothelial dysfunction in kidneys of CRF dogs *in vivo*, with the use of an intravital needle-lens CCD camera technique. The impaired endothelial function in CRF was attributable to the NO synthase inhibition by ADMA, in which the downregulation of an ADMA-degrading enzyme, DDAH, was involved, and possibly to elevated systemic blood pressure. Such deranged NO metabolism would aggravate renal function as well as increase the risk for cardiovascular events in CRF.

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