

Molecular Mechanism for Elevation of Asymmetric Dimethylarginine and Its Role for Hypertension in Chronic Kidney Disease

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Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase. ADMA is generated by protein methyltransferase (PRMT) and is metabolized mainly by dimethylarginine dimethylaminohydrolase (DDAH). ADMA levels are reported to increase in patients with chronic kidney disease (CKD), thereby playing a role in the pathogenesis of accelerated atherosclerosis in this population. However, the precise mechanism underlying ADMA accumulation in these patients is not fully understood. This study investigated the molecular mechanism for the elevation of ADMA levels in CKD, using a rat remnant kidney model that represents progressive CKD. After male Sprague-Dawley rats underwent baseline measurement of BP and renal function, 5/6 subtotal nephrectomy (5/6Nx) and 4/6 nephrectomy were performed. Plasma and urinary levels of ADMA and symmetric dimethylarginine, an inert isomer of ADMA, were measured by HPLC. Expression levels of PRMT genes and DDAH proteins were analyzed by semiquantitative reverse transcription-PCR and Western blotting, respectively. Plasma ADMA levels were elevated in the Nx groups in proportion to the degree of nephrectomy despite marked increases in renal clearance of ADMA. In contrast, renal clearance of symmetric dimethylarginine was decreased and its plasma levels were increased in the Nx groups. Furthermore, both liver and kidney gene expression of PRMT was increased, whereas DDAH protein expression was decreased in the 5/6Nx group. Plasma ADMA levels were correlated with systolic BP levels. Moreover, adenovirus-mediated DDAH gene transfer into the 5/6Nx rats prevented the elevation of BP levels, which was associated with the reduction of plasma and urinary ADMA levels. The results presented here suggest that decreased DDAH levels as well as increased PRMT gene expression could cause the elevation of plasma ADMA levels, thereby eliciting hypertension in CKD. Substitution of DDAH protein or enhancement of its activity may become a novel therapeutic strategy for the treatment of hypertension-related vascular injury in CKD.

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Cardiovascular disease is a major cause of death in patients with ESRD (1). Endothelial dysfunction as a result of reduced bioavailability of nitric oxide (NO) is an initial step of atherosclerosis in patients with chronic kidney disease (CKD) (2,3). NO is synthesized by stereospecific oxidation of the terminal guanidine nitrogen of L-arginine by the action of the NO synthase (NOS). The synthesis of NO can be blocked by inhibition of the NOS active site with guanidino-substituted analogues of L-arginine, such as asymmetric dimethylarginine (ADMA) (2,3). This modified amino acid is delivered from proteins that have been posttranslationally methylated and subsequently hydrolyzed (4). ADMA is formed

by protein methyltransferase (PRMT) (5) and metabolized mainly by N^G,N^G-dimethylarginine dimethylaminohydrolase (DDAH) (6–8).

We, along with others, have demonstrated that high levels of ADMA are observed in hypertension (9), hypercholesterolemia (10), diabetes (11), and CKD (3). Furthermore, we previously showed that there was a close association between plasma ADMA levels and carotid intima-media thickness, one of the surrogate markers of atherosclerosis in the general population (12). In addition, ADMA is a strong prognostic marker of cardiovascular events in patients with coronary artery disease (13), diabetes (14), and ESRD (15). These observations suggest that the elevation of ADMA could contribute to accelerated atherosclerosis by inactivating NOS in CKD. However, the precise molecular mechanism underlying ADMA elevation in this population is not fully understood. Therefore, in this study, we investigated the kinetics of methylated arginines, ADMA and symmetric dimethylarginine (SDMA), an inert isomer of ADMA, and expression levels of PRMT genes and DDAH

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proteins, using a rat remnant kidney model, an experimental model of progressive CKD.

Materials and Methods

Experimental Protocol

After male Sprague-Dawley rats (200 to 250 g) underwent baseline measurement of BP and renal function, 4/6 nephrectomy (4/6Nx; right nephrectomy with surgical resection of the lower third of left kidney) or 5/6 nephrectomy (5/6Nx; right nephrectomy with surgical resection of the lower and upper thirds of left kidney) were performed as described previously (16). Sham-operated rats (sham) underwent the same procedure without the surgical reduction of the kidney. Twelve weeks after the operation, BP was measured using a tail-cuff sphygmomanometer and an automated system with a photoelectric sensor (BP-98A; Softron, Tokyo, Japan). After measurements of BP, the rats were transferred to metabolic cages for 2 d for urinalysis and then killed. All experimental procedures were conducted in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the ethical committee of our institution.

Chemical Analysis

Plasma and urinary levels of SDMA, ADMA, and nitrate and nitrite (NO_x) were measured by HPLC as described previously (8). Creatinine (Cr) and blood urea nitrogen were determined with commercial kits (DENKA SEIKEN Co., Tokyo, Japan, and Alfresa Pharma Co., Osaka, Japan, respectively). Cr clearance (Ccr; ml/min) was determined by the following formula: [urine Cr (mg/dl)] × [urine volume (ml/d)]/[plasma Cr (mg/dl)]/[1440 (min)]. Renal clearance of dimethylarginines (ADMA and SDMA) was calculated by the following formula [urine dimethylarginine (μM)] × [urine volume (ml/d)]/[plasma dimethylarginine (μM)]/[1440 (min)].

Antibodies against DDAH-I and DDAH-II

A mAb against DDAH-I was prepared as described previously (8). A rabbit polyclonal antibody against DDAH-II-specific peptide (LHRGGGDLPN SQE [amino acids 235 to 247]) were prepared by Sigma Genosys (Ishikari, Japan). We confirmed that the antibody against DDAH-II peptide actually binds to a recombinant DDAH-II protein (data not shown).

Western Blot Analysis

The kidney cortex or liver tissues were homogenized (Polytron; Brinkmann Instruments, Westbury, NY) and lysed with 500 μl of 25 mmol/L Tris-HCl (pH 7.4) that contained 1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA, and 1% protease inhibitor cocktail (Nakarai tesque, Kyoto, Japan). Then the supernatant was collected after centrifugation at 14,000 rpm for 30 min at 4°C. Western blot analysis was performed as described previously (17). For confirmation that equal amounts of the proteins were applied for each lane, the membranes were reprobed with an antibody for β-actin (Sigma, St. Louis, MO). Densitometric analysis was performed with National Institutes of Health image software, and the relative ratio to the β-actin expression was calculated in each sample.

Primers and Probes

Primer sequences that were used in semiquantitative reverse transcription-PCR (RT-PCR) were 5'-AACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCCTTGGC-3' for PRMT-1, 5'-TCGGA-CAACCAAGTACCACA-3' and 5'-CCACCTTCTGCTGAAACACA-3' for PRMT-2, and 5'-CCTCAGTGTCTCCAGCCATAGCC-3' and 5'-GGCTATGGCTGGAGAACACTGAGG-3' for PRMT-3. Sequences of

the upstream and downstream primers that were used in semiquantitative RT-PCR for detecting rat glyceraldehyde-3-phosphate dehydrogenase mRNA were the same as described previously (18).

Semiquantitative RT-PCR

Poly(A)⁺RNA were isolated from kidney cortex or liver tissues and analyzed by RT-PCR as described previously (18). The amounts of poly(A)⁺RNA templates (30 ng) and cycle numbers (28 cycles for PRMT-1 gene, 33 cycles for PRMT-2 gene, 32 cycles for PRMT-3 gene, and 22 cycles for glyceraldehyde-3-phosphate dehydrogenase gene) for amplification were chosen in quantitative ranges, where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers (18).

Recombinant Adenoviruses

The plasmid, including the entire coding region of rat DDAH-I cDNA, was cloned as described previously (8). For producing Adv-DDAH, an adenovirus vector that expresses DDAH protein under the control of the cytomegalovirus promoter, the entire coding region of DDAH was inserted into an E1, E3-deleted, human adenovirus serotype 5 mutant, dl7001, with homologous recombination in 293 cells (American Tissue Culture Collection, Bethesda, MD) as described previously (8). Four weeks after 5/6Nx, BP was measured and blood and urine samples were collected. Then the rats were randomly divided into two groups; one was injected with 1.5×10^{10} plaque-forming units of Adv-DDAH through the tail vein, the other with Adv-LacZ, which encodes bacterial β-galactosidase. Determination of infection efficiencies by *in situ* X-Gal staining of the liver revealed that at least 80% of cells were positive for β-galactosidase 14 d after the infection with Adv-LacZ (data not shown). DDAH infection was found actually to increase its level and enzymatic activity in the liver by approximately 2.5-fold compared with that of Adv-LZ-infected rats (data not shown).

Statistical Analyses

All data were expressed as mean ± SE. Experimental groups were compared by ANOVA and, when appropriate, with Scheffe test for multiple comparisons. Linear regression analysis was performed between plasma levels of ADMA and SDMA, Ccr, urinary protein excretion, and total cholesterol levels and BP. A level of *P* < 0.05 was accepted as statistically significant.

Results

Characteristics of the Experimental Model

As shown in Table 1, body weight was significantly lower in the 5/6Nx group compared with the sham and the 4/6Nx groups. Resection of the kidney increased blood urea nitrogen (sham 18.6 ± 0.6 ; 4/6Nx 41.0 ± 7.9 ; 5/6Nx 109 ± 30 mg/dl) and Cr levels (sham 0.34 ± 0.04 ; 4/6Nx 0.85 ± 0.2 ; 5/6Nx 2.2 ± 0.5 mg/dl) and decreased Ccr values (50% decrease in 4/6Nx, 90% decrease in 5/6Nx). Subtotal nephrectomy (4/6Nx and 5/6Nx) also increased systolic BP levels and urinary protein excretions (Table 1).

Plasma and Urine Levels of NO_x, LArginine, and Methylated Arginines

As shown in Table 2, plasma ADMA and SDMA were increased in proportion to the degree of nephrectomy. Renal clearance of ADMA was markedly increased in the Nx groups, whereas that of SDMA was decreased. Furthermore, urinary excretion of NO_x was significantly decreased in proportion to

Table 1. Clinical variables^a

	Body Weight (g)	BUN (mg/dl)	Cr (mg/dl)	Ccr (ml/min)	SBP (mmHg)	UP (g/g Cr)
Sham (<i>n</i> = 9)	617 ± 19	18.6 ± 0.6	0.34 ± 0.04	3.8 ± 0.3	122 ± 4	0.8 ± 0.1
4/6Nx (<i>n</i> = 7)	583 ± 32	41.0 ± 7.9 ^b	0.85 ± 0.2 ^b	1.9 ± 0.3 ^b	153 ± 7 ^b	7.2 ± 2.7 ^b
5/6Nx (<i>n</i> = 6)	492 ± 35 ^b	109 ± 30 ^{b,c}	2.2 ± 0.5 ^{b,c}	0.5 ± 0.2 ^{b,c}	199 ± 10 ^{b,c}	39 ± 11 ^{b,c}

^aData are mean ± SEM. 4/6Nx, 4/6 nephrectomy rats; 5/6Nx, 5/6 nephrectomy rats; BUN, blood urea nitrogen; and Ccr, 24-h creatinine clearance; Cr, serum creatinine; SBP, systolic BP; sham, sham-operated rats; UP, urinary protein excretion.

^b*P* < 0.05 compared with the value of the sham-operated rats.

^c*P* < 0.05 compared with the value of the 4/6Nx rats.

Table 2. Plasma and urinary excretion levels of methylated arginines and their renal clearance^a

	Plasma (μM)		Urinary Excretion (μmol/g Cr)		Renal Clearance (ml/min)	
	ADMA	SDMA	ADMA	SDMA	ADMA	SDMA
Sham (<i>n</i> = 9)	0.49 ± 0.02	0.29 ± 0.02	1.4 ± 0.3	31 ± 2.8	0.04 ± 0.01	1.4 ± 0.13
4/6Nx (<i>n</i> = 7)	0.62 ± 0.02 ^b	0.53 ± 0.07 ^b	8.3 ± 1.6 ^b	28 ± 3.3	0.16 ± 0.03 ^b	0.75 ± 0.12 ^b
5/6Nx (<i>n</i> = 6)	0.71 ± 0.05 ^{b,c}	1.1 ± 0.2 ^{b,c}	11.4 ± 2.7 ^{b,c}	28 ± 5.5	0.14 ± 0.04 ^b	0.23 ± 0.05 ^{b,c}

^aData are mean ± SEM. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.

^b*P* < 0.05 compared with the value of the sham-operated rats.

^c*P* < 0.05 compared with the value of the 4/6Nx rats.

the degree of nephrectomy (sham 11.6 ± 2.1; 4/6Nx 6.4 ± 2.7; 5/6Nx 2.4 ± 0.9 μmol/d; *P* < 0.01), whereas the plasma levels of L-arginine, a NO substrate, were similar among the three groups (sham 192 ± 44; 4/6Nx 188 ± 24; 5/6Nx 165 ± 7.2 μmol/L; NS). Plasma levels of ADMA were negatively correlated to urinary excretion levels of NOx (*r*² = 0.2, *P* < 0.05).

ADMA-Related Enzyme Expression in Both Kidney and Liver

Because impaired renal clearance of ADMA was not a cause of ADMA accumulation in this model, we next examined expression levels of PRMT genes and DDAH proteins. As shown in Figure 1, A and B, renal and liver gene expression levels of type I PRMT (PRMT-1 and PRMT-3), which catalyze the asymmetric dimethylation and monomethylation of arginine residues and resultantly produces ADMA and N^G-monomethyl-L-arginine (5), were significantly increased in the 5/6Nx group. The level of type II PRMT (PRMT-2), which produces SDMA and N^G-monomethyl-L-arginine but not ADMA (5), had a tendency to increase in the 5/6Nx group (Figure 1A).

It has been reported that two isoforms of DDAH (DDAH-I and DDAH-II) are expressed in both kidney and liver (19). Therefore, we next investigated the expression levels of DDAH in our model. As shown in Figure 1, C and D, the renal and liver expression levels of DDAH-I and DDAH-II were significantly decreased in the 5/6Nx group. As shown in Figure 2, the changes in DDAH and PRMT levels were observed at an early phase of nephrectomy. In parallel to these alterations, urinary ADMA excretion was found to be increased.

ADMA and BP

To assess the pathologic role for ADMA in CKD, we next investigated the relationship between ADMA and BP. By linear

regression analysis, plasma levels of ADMA (Figure 3A) and SDMA (Figure 3B), Ccr (*r*² = 0.61, *P* < 0.0001), urinary protein excretion (*r*² = 0.55, *P* = 0.0003), and total cholesterol levels (*r*² = 0.55, *P* = 0.0002) were found to correlate to BP. Multiple stepwise regression analysis revealed that Ccr was a sole determinant for BP (data not shown). Ccr was more strongly and negatively correlated with plasma levels of SDMA (*r*² = 0.66, *P* < 0.01) than those of ADMA (*r*² = 0.33, *P* < 0.01). Therefore, to investigate the direct involvement of ADMA in BP, we examined the effect of DDAH overexpression on BP in our model. DDAH infection significantly reduced plasma and urinary ADMA levels (plasma levels of ADMA before infection 0.64 ± 0.05 μM and 14 d after infection 0.56 ± 0.03 μM [*P* < 0.05]; urinary levels of ADMA before infection 7.6 ± 1.6 μmol/g Cr and 14 d after infection 2.2 ± 0.9 μmol/g Cr [*P* < 0.01]) and subsequently prevented the elevation of BP in the 5/6Nx rats (Figure 4). It has been reported that mice that overexpress DDAH have a higher heart rate (20), whereas infusion of ADMA into humans lowers the heart rate (21). However, the heart rate was not changed during the experimental periods in this study (before infection 385 ± 13 bpm; 14 d after infection 379 ± 10 bpm).

Discussion

The salient findings of this study were that (1) renal excretion of ADMA was increased rather than decreased; (2) PRMT, which is an enzyme to synthesize ADMA, was increased and DDAH, which is a key-limiting enzyme to metabolize ADMA but not SDMA, was decreased; (3) plasma levels of ADMA were correlated with BP levels; and (4) DDAH overexpression decreased plasma levels of ADMA and subsequently prevented the elevation of BP levels in the animal model of CKD.

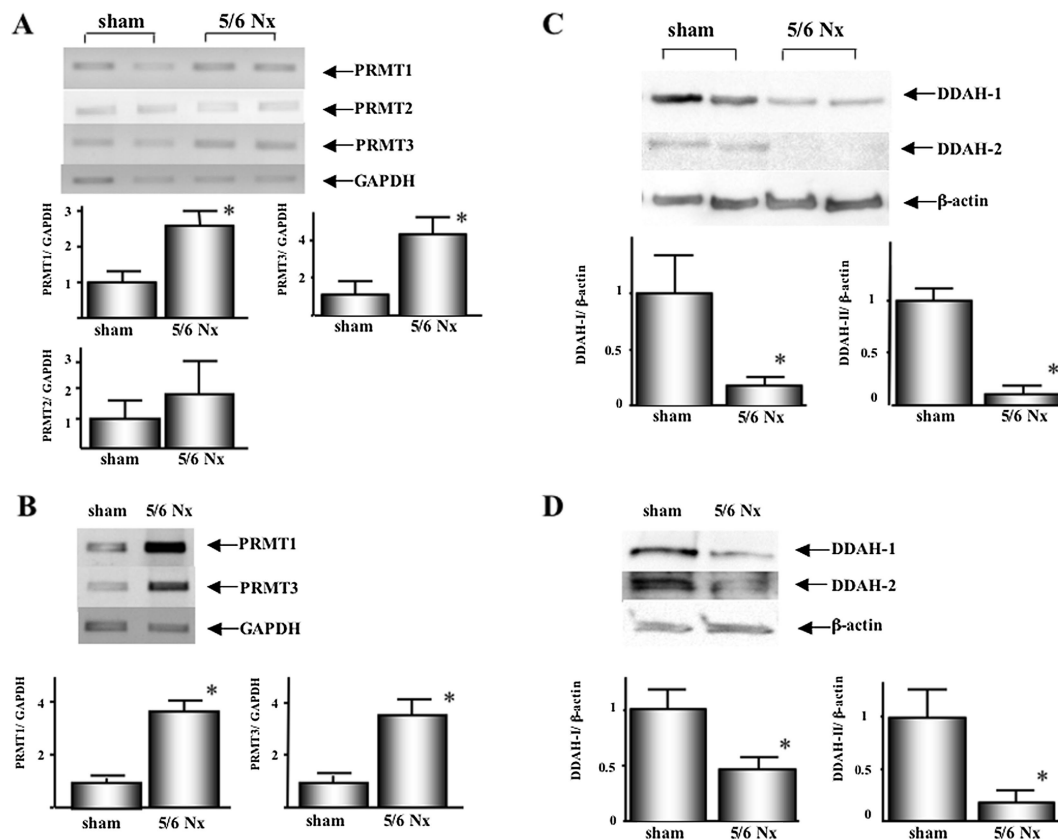


Figure 1. Protein methyltransferase (PRMT) gene and dimethylarginine dimethylaminohydrolase (DDAH) protein levels 12 wk after nephrectomy. (Top) Representative reverse transcription–PCR (RT-PCR) bands of PRMT in kidney (A) and liver (B) and Western blots of DDAH proteins in kidney (C) and liver (D). (Bottom) Quantitative representation of PRMT genes and DDAH proteins. Data were normalized by the intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene or β-actin protein and were expressed as mean ± SE (*n* = 6). **P* < 0.05 compared with the value of the sham-operated rats.

There is a growing body of evidence to show that ADMA is implicated in the pathogenesis of atherosclerosis (2,12). Indeed, plasma ADMA levels are elevated in patients with CKD (3,22–24) and are a strong predictor for cardiovascular disease and mortality in these patients (15,25). Until now, it was assumed that impaired urinary clearance could account, at least in a part, for the elevation of ADMA level in CKD. However, in this study, we demonstrated that urinary clearance of ADMA was increased rather than decreased in the Nx groups than in the sham-operated group. In contrast, renal clearance of SDMA was disturbed in the Nx groups. These observations suggest that impaired renal clearance of SDMA could be a main cause of the elevation of this methylated arginine, whereas that of ADMA cannot explain its elevation in our CKD model. It has been reported that when dimethylarginines are injected intravenously, 66% of SDMA is recovered in the urine, whereas only 5% of ADMA is recovered in the urine (26). These observations suggest that SDMA could be almost entirely excreted by kidney, whereas ADMA could be metabolized extensively rather than excreted in urine. Similar findings were reported by Banachabouchi *et al.* (27). In their study, ADMA levels in 80% nephrectomized rats increased not only in the plasma but also in the urine; urinary ADMA levels increased up to eight-fold, but they also showed that patients with CKD and nephrecto-

mized mice had a decreased renal clearance of ADMA, suggesting marked differences of urinary clearance of ADMA between species.

To investigate further the molecular mechanism for the elevation of ADMA, we next examined expression levels of PRMT genes and DDAH proteins. In this study, we found that expression levels of PRMT-1 and PRMT-3 were increased in the 5/6Nx group. These findings suggest that enhancement of ADMA production could be one possible mechanism for the elevation of ADMA in our CKD model. In support of our findings, Okubo *et al.* (28) demonstrated that inhibition of PRMT by adenosine dialdehyde decreased plasma ADMA levels in an experimental model of ESRD. Furthermore, we demonstrated here that DDAH-I and DDAH-II protein expressions were significantly decreased in the 5/6Nx group. These observations suggest that impaired metabolism of ADMA as a result of reduced DDAH levels also may be a causal factor for the elevation of ADMA in this model. More than 90% of ADMA is eliminated by the action of DDAH in rats (29). In addition, the impaired ability of DDAH was associated with ADMA accumulation in diabetic rats or hypercholesterolemic rabbits without renal dysfunction (11,30). These findings strongly support that decreased levels or activity of DDAH also could contribute to the elevation of ADMA levels in CKD.

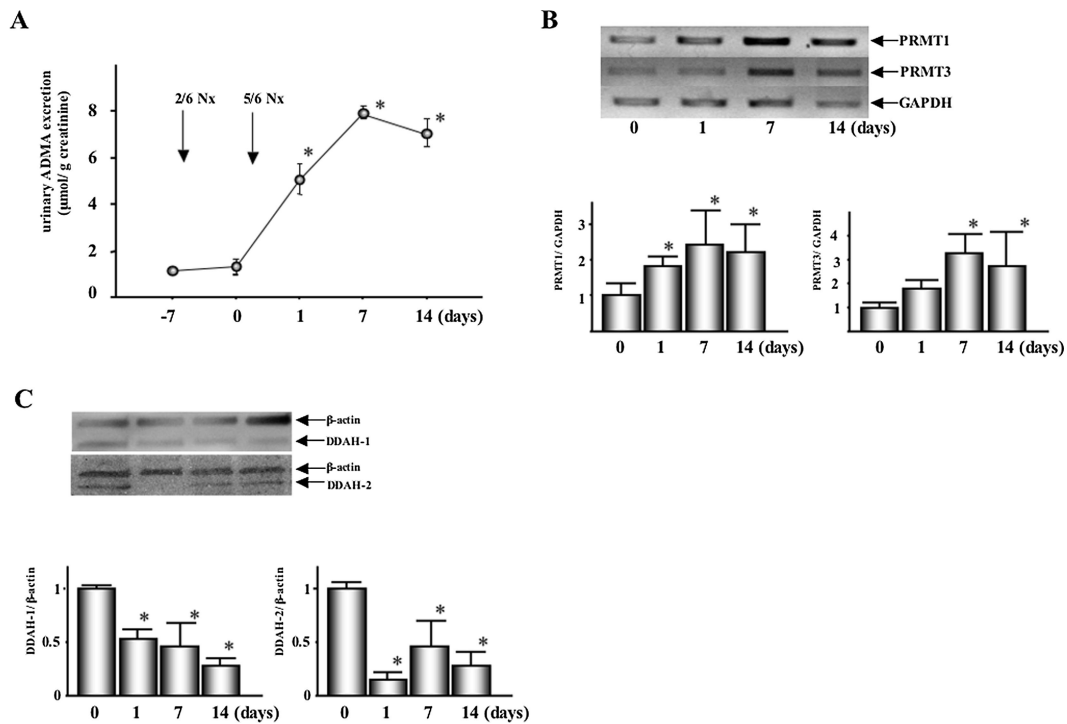


Figure 2. Time course of changes in PRMT gene and DDAH protein expression after nephrectomy. (A) Levels of urinary asymmetric dimethylarginine (ADMA) excretion were measured on the indicated days ($n = 5$). Data were normalized by the concentration of urinary creatinine. * $P < 0.01$ compared with the value of the rats before nephrectomy. (B, top) Representative RT-PCR bands of PRMT in kidney. (B, bottom) Quantitative representation of PRMT genes. (C, top) Western blots of DDAH proteins in kidney. (C, bottom) Quantitative representation of DDAH proteins. Data were normalized by the intensity of GAPDH gene or β -actin protein and were expressed as mean \pm SE ($n = 5$). * $P < 0.05$ compared with the value of the rats before nephrectomy.

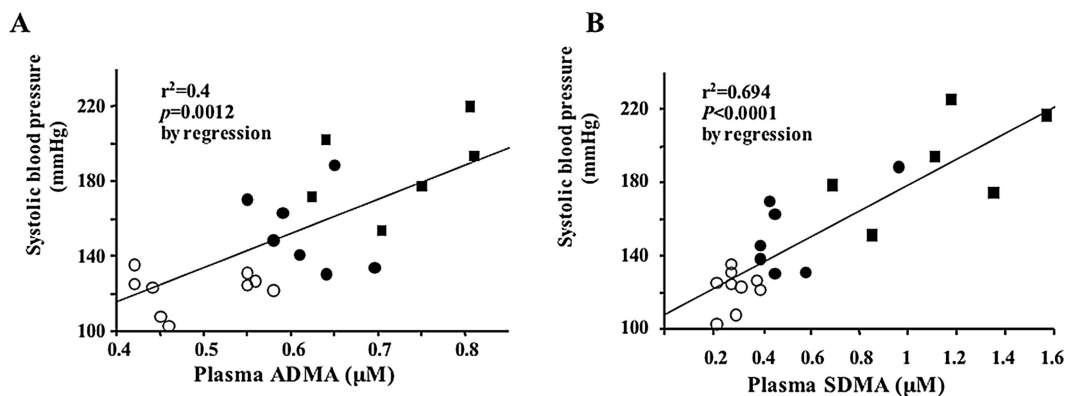


Figure 3. Correlations between systolic BP and plasma levels of ADMA (A) and symmetric dimethylarginine (SDMA; B). sham, sham-operated rats (\circ ; $n = 9$), 4/6Nx; 4/6 nephrectomy rats (\bullet ; $n = 7$), 5/6Nx; 5/6 nephrectomy rats (\blacksquare ; $n = 6$).

In this study, we cannot clarify the molecular mechanisms of upregulation of PRMT and downregulation of DDAH in our CKD model. However, we speculate that oxidative stress could be involved in the dysregulation of PRMT and DDAH by the following evidence: (1) Gene expressions of type I PRMT were increased by oxidized LDL in cultured endothelial cells (EC) through a redox-regulated mechanism (5); (2) DDAH activities in EC and smooth muscle cells were reduced under high glucose conditions, which were prevented by an antioxidant, poly-

ethylene glycol-conjugated superoxide dismutase (11); and (3) it is widely known that oxidative stress generation is increased in patients with CKD (31,32). Uremia-related oxidative stress as well as uremic toxins such as homocysteine and advanced glycation end products, which decrease DDAH activity (33,34), may have contributed to dysregulation of these enzymes. Furthermore, recently, coupling factor 6, an endogenous inhibitor of prostacyclin synthesis, was found to be elevated in patients with ESRD and positively associated with plasma levels of

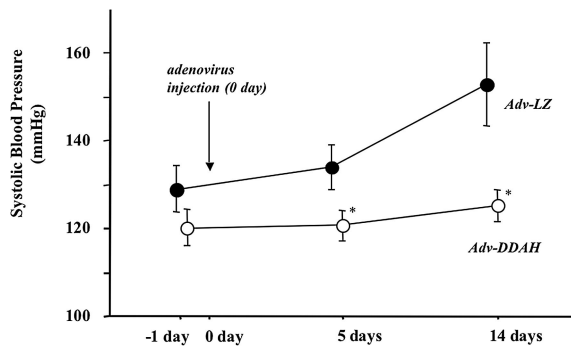


Figure 4. Effect of DDAH overexpression on BP in the remnant kidney model. Four weeks after the 5/6Nx, adenovirus vector encoding DDAH (Adv-DDAH; ○; $n = 18$) or encoding β -galactosidase (Adv-LZ; ●; $n = 12$) were injected into the rats. Then BP was measured on the indicated days. * $P < 0.05$ compared with the value of the Adv-LZ.

ADMA (35). In addition, coupling factor 6 increased EC gene expression of PRMT, whereas it decreased that of DDAH (36). Taken together, oxidative stress and/or coupling factor 6 could be involved in dysregulation of PRMT and DDAH in our models. Moreover, in this study, the time-course experiments (Figure 2) revealed that the alterations of DDAH and PRMT levels were observed at early phase of nephrectomy, whose changes were parallel to the increase in urinary ADMA excretion. These results suggest that early hemodynamic changes after nephrectomy also may affect the levels of DDAH and PRMT. We also do not know the mechanisms by which renal resection increased urinary ADMA excretion in this study. However, it is possible that the increased urinary excretion was due to the systemic accumulation of ADMA by overproduction and decreased degradation of ADMA. In this study, DDAH overexpression reduced urinary excretion of ADMA, also suggesting decreased extrarenal clearance of ADMA.

Hypertension is the most common complication in patients with CKD and is not only a predictor of mortality of cardiovascular complications but also a determinant for progression of renal disease (37). In this study, plasma levels of ADMA were correlated to BP levels. Furthermore, DDAH overexpression decreased plasma levels of ADMA and subsequently prevented the elevation of BP levels. Moreover, Dayoub and colleagues (20,38) recently reported that BP is significantly lower in DDAH-transgenic mice than in wild-type mice. These observations suggest the pathologic role for the increase of plasma ADMA levels in the elevation of BP in CKD. However, several papers showed no correlation between ADMA and BP (39,40). Other factors than ADMA have been known to participate in the BP elevation after subtotal nephrectomy, such as angiotensin II (41). Furthermore, several factors, such as age, insulin resistance, and dyslipidemia, also could affect the plasma levels of ADMA (5,10,12,30,42). These confounding factors and/or cardiac medications might attenuate the positive correlation between plasma ADMA levels and BP, which could explain the negative clinical studies.

Conclusion

Decreased DDAH as well as increased PRMT expression but not impaired renal clearance of ADMA could cause the elevation of plasma ADMA levels, thereby eliciting BP elevation in CKD. Substitution of DDAH or enhancement of its activity may become a novel therapeutic strategy for the treatment of hypertension-associated vascular injury in CKD.

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