Dimethylarginine Dimethylaminohydrolase Prevents Progression of Renal Dysfunction by Inhibiting Loss of Peritubular Capillaries and Tubulointerstitial Fibrosis in a Rat Model of Chronic Kidney Disease

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Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor, is mainly degraded by dimethylarginine dimethylaminohydrolase (DDAH). It was recently reported that reduced DDAH expression could contribute to ADMA accumulation and subsequent elevation of BP in an experimental model of chronic kidney disease (CKD). ADMA is a strong predictor of the progression of CKD as well. However, a role for the ADMA-DDAH in the pathogenesis of CKD remains to be elucidated. This study investigated the effects of DDAH-elicited ADMA lowering on renal function and pathology in a rat remnant kidney model. Four weeks after five-sixths subtotal nephrectomy (Nx), the rats were given tail-vein injections of recombinant adenovirus vector encoding DDAH-I (Adv-DDAH) or control vector expressing bacterial β-galactosidase (Adv-LZ) or orally administered 20 mg/kg per d hydralazine (Hyz), which served as a BP control model. In comparison with Adv-LZ or Hyz administration, Adv-DDAH decreased plasma levels of ADMA and inhibited the deterioration of renal dysfunction. Plasma levels of ADMA were associated with decreased number of peritubular capillaries, increased tubulointerstitial fibrosis, and proteinuria levels inNx rats. These changes were progressed in Adv-LZ– or Hyz-treated Nx rats, which were ameliorated by DDAH overexpression. In addition, semiquantitative reverse transcriptase–PCR and immunohistochemistry for TGF-β revealed that Adv-DDAH inhibited upregulation of TGF-β expression in Nx rats. These data suggest that ADMA may be involved in peritubular capillary loss and tubulointerstitial fibrosis, thereby contributing to the progression of CKD. Substitution of DDAH protein or enhancement of its activity may become a novel therapeutic strategy for the treatment of CKD.


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Nitric oxide (NO) is synthesized by stereospecific oxidation of 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) (1,2). We, along with others, have demonstrated that elevated plasma ADMA is associated with cardiovascular risk factors such as hypertension (3,4), diabetes (4,5), and chronic kidney disease (CKD) (6,7), thereby being one of the useful biomarkers for atherosclerosis and future cardiovascular events (5,7–9). ADMA is mainly metabolized by an enzyme dimethylarginine dimethylaminohydrolase (DDAH) (1,2). We recently found that reduced DDAH expression could contribute to ADMA accumulation and subsequent elevation of BP in an experimental model of CKD (10). Furthermore, plasma level of ADMA is known to be a strong predictor of the progression of renal dysfunction in patients with CKD (11,12). According to the recent comprehensive review on the potential role of ADMA in renal disease progression (13), there may be two major possible mechanisms by which ADMA could contribute to the progression of CKD; one is a BP-dependent effect of ADMA described previously (4,10), and the other is a BP-independent, direct effect of ADMA on renal microvasculature. As to the latter, Kang et al. (14) demonstrated that administration of an inhibitor of NOS accelerated renal injury and impaired angiogenic response and peritubular capillary formation in the remnant kidney model, whose harmful effects were greater than expected from the increase in BP levels, thus suggesting that an important role of NO in maintaining renal microvasculature (15,16). Therefore, it is plausible that DDAH could protect against renal damage by suppressing the inhibitory effect of ADMA on NO generation. In this study, we investigated the effects of DDAH-elicited ADMA lowering on renal function and tubulointerstitial changes in a rat remnant kidney model. To address the issue of whether DDAH could protect against renal injury in a BP-independent manner, we compared the renoprotective effects of DDAH with those of hydralazine (Hyz), an antihypertensive drug with equihypotensive property.
that were treated with hydralazine. Adv-DDAH, Nx rats that were treated with adenovirus encoding dimethylarginine dimethylaminohydrolase; Hyz, Nx rats that were treated with adenovirus encoding β-galactosidase (Adv-LZ)-treated diabetic rats.

Materials and Methods
Recombinant Adenoviruses
The plasmid, including the entire coding region of DDAH-I cDNA, was cloned as described previously (17). To produce adenovirus vector encoding human DDAH-I protein under the control of the cytomegalovirus promoter (Adv-DDAH), 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 (17).

Experimental Protocol
After male Sprague-Dawley rats (200 to 250 g) underwent baseline measurement of BP and renal function, five-sixths subtotal nephrectomy (Nx; right nephrectomy with surgical resection of the lower and upper thirds of left kidney) was performed as described previously (10). Four weeks after Nx, BP was measured using a tail-cuff sphygmomanometer using an automated system with a photoelectric sensor (BP-98A; Softron, Tokyo, Japan), and blood and urine samples were collected. Then, the rats were randomly divided into four groups: Rats that were immediately killed (n = 15) or control adenovirus vector–expressing bacterial β-galactosidase (Adv-LZ; n = 15); and rats that were orally administered 20 mg/kg per d Hyz, which served as a BP control model (n = 6). Fourteen days after the treatment, BP was measured and the rats were transferred to metabolic cages for 2 d for urinalysis. Then, the rats were killed. All experimental procedures were conducted in accordance 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 levels of l-arginine, ADMA, and symmetric dimethylarginine (SDMA), an inert isomer of ADMA, were measured by HPLC as described previously (10,17). Serum level of creatinine (cr) was determined with commercial kits (Denka Seiken, Co., Tokyo, Japan). Creatinine clearance (Ccr, ml/min) was determined as the following formula: [urine creatinine (mg/dl)] × [urine volume (ml/d)]/[plasma creatinine (mg/dl)]/[1440 (min)].

Immunohistochemistry
The kidneys were removed and fixed in 4% paraformaldehyde. Then the kidneys were embedded in paraffin wax for sectioning. Three-micrometer paraffin sections were incubated with monoclonal IgG-JG-12 antibody raised against anionic substrates (Bender MedSystems, San Bruno, CA) or polyclonal antibody raised against TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA). After exposure to peroxidase-labeled secondary anti-mouse antibody, the sections were incubated with 3,3′-diaminobenzidine solution. The intensity of JG-12 or TGF-β staining was analyzed by an image analysis software (Optimas version 6.57; Media Cybernetics, Silver Spring, MD).

Renal Histology Analysis
Three-micrometer paraffin sections were stained with Azan to evaluate interstitial fibrosis. The intensity of the positive for Azan was also quantified by computer image analysis (Optimas version 6.57).

Primers
Primer sequences that were used in semiquantitative reverse transcription–PCR (RT-PCR) were 5′-AGACACCGCAGTCTTCTGT-3′ and 5′-CCACAGTCTGAGTGCA-3′ for glyceraldehyde-3-

### Table 1. l-arginine and dimethylarginine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>l-Arginine (µM)</th>
<th>ADMA (µM)</th>
<th>SDMA (µM)</th>
<th>l-Arginine/ADMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adv-LZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>80.8 (29.9 to 126.1)</td>
<td>0.70 (0.59 to 0.87)</td>
<td>0.82 (0.66 to 1.06)</td>
<td>104.1 (45.3 to 185.4)</td>
</tr>
<tr>
<td>after</td>
<td>64.6 (25.6 to 149.4)</td>
<td>0.73 (0.56 to 0.98)</td>
<td>0.84 (0.62 to 1.56)</td>
<td>86.3 (31.6 to 266.8)</td>
</tr>
<tr>
<td>Adv-DDAH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>64.2 (33.5 to 128.4)</td>
<td>0.73 (0.45 to 0.98)</td>
<td>0.80 (0.74 to 1.02)</td>
<td>88.9 (42.9 to 173.5)</td>
</tr>
<tr>
<td>after</td>
<td>62.6 (13.6 to 153.8)</td>
<td>0.52 (0.40 to 0.74)</td>
<td>0.84 (0.70 to 1.05)</td>
<td>118.0 (24.3 to 334.4)</td>
</tr>
<tr>
<td>Hyz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>69.4 (49.9 to 113.2)</td>
<td>0.71 (0.54 to 0.95)</td>
<td>0.84 (0.6 to 1.41)</td>
<td>81.6 (58.0 to 182.6)</td>
</tr>
<tr>
<td>after</td>
<td>71.1 (25.6 to 149.4)</td>
<td>0.74 (0.63 to 0.99)</td>
<td>0.82 (0.69 to 1.60)</td>
<td>83.8 (50.1 to 158.0)</td>
</tr>
</tbody>
</table>

aData are median (range). Adv-LZ, nephrectomized (Nx) rats that were treated with adenovirus encoding β-galactosidase; Adv-DDAH, Nx rats that were treated with adenovirus encoding dimethylarginine dimethylaminohydrolase; Hyz, Nx rats that were treated with hydralazine.

bP < 0.05 versus rats before treatment.
cP < 0.05 versus Adv-LZ.
Table 2. Baseline clinical variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>cr</th>
<th>Ccr</th>
<th>UP</th>
<th>SBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adv-LZ</td>
<td>15</td>
<td>0.94 (0.71 to 1.46)</td>
<td>0.91 (0.66 to 1.20)</td>
<td>6.0 (2.0 to 24.9)</td>
<td>132 (110 to 142)</td>
</tr>
<tr>
<td>Adv-DDAH</td>
<td>15</td>
<td>0.93 (0.56 to 1.41)</td>
<td>1.03 (0.51 to 1.28)</td>
<td>4.7 (1.7 to 16.7)</td>
<td>122 (91 to 155)</td>
</tr>
<tr>
<td>Hyz</td>
<td>6</td>
<td>0.98 (0.72 to 1.31)</td>
<td>0.92 (0.63 to 1.47)</td>
<td>5.5 (2.5 to 8.1)</td>
<td>128 (121 to 135)</td>
</tr>
</tbody>
</table>

Data are median (range). Ccr, creatinine clearance (ml/min); UP, urinary protein excretion (g/g creatinine); SBP, systolic blood pressure (mmHg).

DDAH Activity

Total DDAH activity was measured as described previously (17). Briefly, homogenized liver tissue was incubated with 4 μmol/L ADMA and 0.1 mol/L sodium phosphate buffer (pH 6.5) in a total volume of 0.5 ml for 6 h at 37°C. The reaction was stopped by the addition of equal volume of 10% TCA, and the supernatant was boiled with diacetyl monoxime (0.8% [wt/vol] in 5% acetic acid) and antipyrine (0.5% [wt/vol] in 50% sulfuric acid). The amounts of L-citrulline formed were determined with the spectrophotometric analysis at 466 nm.

Results

DDAH Gene Transfer

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-LZ (data not shown). DDAH infection was found actually to increase its mRNA level and enzymatic activity in the liver by approximately 2.5-fold compared with that of Adv-LZ–infected rats (Figure 1). Adv-DDAH but not Adv-LZ or Hyz significantly decreased plasma levels of ADMA (Table 1). Although each treatment did not affect plasma levels of l-arginine or SDMA, l-arginine/ADMA ratio, which was considered to be a good marker for NO production capacity (1,19), was significantly increased by the treatment with DDAH overexpression and tended to be higher than that in Adv-LZ–treated rats.

**Data of Clinical Variables**

As shown in Table 2, baseline renal function (cr and Ccr), urinary excretion of protein (UP), and BP were similar among the three groups. As shown in Figure 2, A and B, systolic BP (SBP) as well as mean BP were progressively elevated in Adv-LZ–transfected Nx rats (SBP levels: before infection 130.4 ± 7.7 mmHg, 14 d after infection 153.9 ± 15.9 mmHg; mean BP: levels before infection 104.6 ± 3.5 mmHg, 14 d after infection 122.8 ± 8.7 mmHg), whereas that of Adv-DDAH–transfected Nx rats remained unchanged (SBP levels: before infection 119 ± 4.3 mmHg, 14 d after infection 122 ± 3.8 mmHg; mean BP: levels: before infection 96.8 ± 4.4 mmHg, 14 d after infection 99.2 ± 4.0 mmHg). Hyz treatment also prevented the elevation of BP levels in Nx rats (SBP levels: before treatment 126.9 ± 2.2 mmHg, 14 d after treatment 105.8 ± 10.3 mmHg; mean BP levels: before treatment 98.2 ± 1.4 mmHg, 14 d after treatment 88.3 ± 4.1 mmHg).

**Dimethylarginine and Renal Function**

A simple correlation analysis revealed that baseline Ccr value was inversely correlated to plasma levels of SDMA ($r^2 = -0.56$, $P < 0.01$) and ADMA ($r^2 = -0.20$, $P < 0.05$) in our model. These findings suggest that SDMA is also a good marker of renal function in rats as described previously (20–22). Furthermore, compared with Adv-LZ (Ccr value: before infection 0.93 ± 0.07 

ml/min, 14 d after infection 0.77 ± 0.09 ml/min) or Hyz treatment (Ccr value: before treatment 0.98 ± 0.16 ml/min, 14 d after treatment 0.87 ± 0.12 ml/min), Adv-DDAH prevented the decrease of Ccr (Ccr value: before infection 1.04 ± 0.09 ml/min, 14 d after infection 1.20 ± 0.14 ml/min) in Nx rats (Figure 2C).

**Relationship between Plasma ADMA Levels and Tubulointerstitial Changes**

NO inhibition progresses renal damage in a remnant kidney model by impairing angiogenesis and subsequently causing fibrosis in tubulointerstitial regions (14,23,24). Therefore, we investigated the relationship between plasma ADMA levels and tubulointerstitial changes in Nx rats. As shown in Figure 3, plasma levels of ADMA were associated with decreased number of peritubular capillaries (Figure 3A) and increased tubulointerstitial fibrosis in Nx rats (Figure 3B).

**Effects of DDAH Overexpression on Tubulointerstitial Changes**

We next investigated the ADMA-lowering effects of Adv-DDAH on tubulointerstitial changes in Nx rats. As shown in Figures 4 and 5, progressive loss of peritubular capillaries and increase in tubulointerstitial fibrosis were observed in Adv-LZ– or Hyz-treated Nx rats, which were ameliorated by the treatment with Adv-DDAH.

**Effects of DDAH Overexpression on Renal TGF-β Gene Expression**

Because TGF-β is recognized as a major causative factor for the pathogenesis of tubulointerstitial fibrosis in CKD (25,26), we next examined the effect of DDAH overexpression on TGF-β expression in Nx rats. Semiquantitative RT-PCR analysis revealed that TGF-β gene expression was increased in Adv-LZ– or Hyz-treated Nx rats, which was significantly reduced by Adv-DDAH (Figure 6A). Furthermore, immunohistochemistry for TGF-β revealed that tubular protein expression levels of
TGF-β/H9252 were also increased in Nx rats, which was blocked by DDAH overexpression but not Hyz (Figure 6B). Effects of DDAH Overexpression on Proteinuria Levels

Recently, Caglar et al. (27) showed that ADMA correlates with proteinuria in patients with CKD stage 1. Therefore, we next investigated whether this is also the case in our experimental model. As shown in Figure 7A, plasma levels of ADMA were positively correlated to urinary protein excretion levels. Furthermore, DDAH overexpression but not Adv-LZ or Hyz administration prevented the increase in urinary protein excretion levels (Figure 7B). These observations suggest that ADMA may play a role in the progression of proteinuria in our CKD model.

Discussion

The salient findings of this study were (1) overexpression of DDAH-I, a rate-limiting enzyme for the degradation of ADMA, significantly decreased plasma levels of ADMA and subsequently blocked the progression of renal dysfunction in the remnant kidney model (Nx rats); (2) plasma levels of ADMA were associated with decreased number of peritubular capillaries and increased tubulointerstitial fibrosis in Nx rats; (3) DDAH overexpression prevented the progressive loss of peritubular capillaries and increase in interstitial fibrosis; (4) DDAH overexpression suppressed TGF-β gene and protein expression in an experimental model of CKD; (5) plasma levels of ADMA were positively correlated with proteinuria; and (6) DDAH overexpression significantly prevented the increase in urinary protein excretion levels. Although Hyz treatment blocked the elevation of BP, it did not decrease plasma ADMA level or improve tubulointerstitial changes, renal dysfunction, or urinary protein excretion levels. Therefore, these observations suggest that DDAH could block the progression of renal damage in CKD by reducing plasma ADMA but not BP levels.

The remnant kidney model is widely considered to be the classic model of CKD. Previous studies have reported that the blockade of NO production in this model is associated with increased systemic and glomerular pressure and loss of peritubular capillaries, thereby resulting in a more rapid course of renal scarring (14). Because ADMA is an endogenous NOS inhibitor and its plasma level is elevated in patients with CKD (6,13,28,29), our study suggests that DDAH overexpression may have a protective role against the progression of renal damage in the remnant kidney model by suppressing the inhibitory effect of ADMA on NO generation. These findings that plasma levels of ADMA were associated with tubulointerstitial changes in Nx rats and that Adv-DDAH but not Adv-LZ or Hyz decreased plasma ADMA levels and ameliorated tubulointerstitial changes, renal dysfunction, and proteinuria further support the pathologic role for the ADMA-DDAH system in the progression of CKD. Although a couple of articles suggested that SDMA also could be implicated in NOS inhibition indirectly by interfering in l-arginine transport (21,30), it is unlikely that DDAH overexpression exerted renoprotective and antihypertensive effects in our CKD model via modulation of SDMA levels because only ADMA but not SDMA is metabolized by DDAH. We confirmed here that DDAH overexpression did not affect plasma SDMA levels in our model (Table 1).

In our CKD model, DDAH overexpression significantly reduced both systolic and mean BP levels. It was in contrast with the previous findings that DDAH I transgenic mice exhibited no difference in mean BP levels, only in SBP levels (31).
though we do not know the exact reason for the discrepant results between ours and theirs, the differences of animal species and models and the different method of DDAH overexpression could partly explain the discrepancy.

It has been recognized that proximal tubular cell atrophy and tubulointerstitial fibrosis are more important than glomerulopathy in terms of renal prognosis in CKD (32,33). Peritubular capillary loss may be involved in chronic ischemia and hypoxia in tubulointerstitium, which could stimulate the renal scarring process (32,33). Indeed, a close relationship between progressive interstitial fibrosis and the loss of peritubular capillary was shown in patients with CKD and animal models of CKD (14,34). Because NO inhibits apoptosis of endothelial cells (35) and increases their proliferation and migration (36,37), DDAH could block the progression of renal damage in Nx rats by maintaining the number of peritubular capillaries. In support of this, DDAH transfection into cultured endothelial cells enhances vascular endo-

Figure 6. Effect of DDAH overexpression on TGF-β expression. (A, top) Representative results of RT-PCR bands of TGF-β gene. (A, bottom) Quantitative representation of TGF-β gene induction. Data were normalized by the intensity of GAPDH mRNA (n = 5, respectively). (B) Representative microphotographs of TGF-β immunostaining: before treatment (a), Adv-LZ–treated rats (b), Adv-DDAH–treated rats (c), and Hyz-treated rats (d). (e) Quantitative analysis of TGF-β immunostaining. *P < 0.05 versus rats before treatment; **P < 0.01 versus Adv-LZ or Hyz. Magnification, ×200.

Figure 7. (A) Correlation between plasma levels of ADMA and urinary protein excretion levels in Sprague-Dawley rats 4 wk after Nx (n = 47). (B) Effects of DDAH overexpression on urinary protein excretion levels. Data are means ± SEM. *P < 0.05 versus Adv-LZ or Hyz.
thelial growth factor mRNA expression and stimulates tube formation of these cells (38). Transfection of DDAH into a glioma tumor cell line promotes tumor angiogenesis and growth in vivo (39). Moreover, in a murine model of hind-limb ischemia, enhanced neovascularization and limb perfusion were observed in DDAH transgenic mice, which were associated with reduced plasma levels of ADMA (40). These observations suggest that the ADMA-DDAH axis may regulate angionic responses in various diseases.

It has been reported that TGF-β gene is upregulated under hypoxic conditions (41). Therefore, the ADMA-elicited peritubular capillary loss could cause tubulointerstitial ischemia and subsequently enhance TGF-β expression in Nx rats. Because several pieces of evidence have implicated TGF-β as a major causative agent in the pathogenesis of tubulointerstitial fibrosis in CKD (25,26), DDAH may ameliorate tubulointerstitial fibrosis in the remnant kidney model by suppressing tubular ischemia and TGF-β overexpression. Recently, blockade of NO synthesis or NO deficiency was reported to promote cardiac or renal fibrosis, respectively, via induction of TGF-β (24,42), thus suggesting the pathologic role for the ADMA-elicited NO reduction in fibrosis in various devastating disorders.

In our CKD model, ADMA levels were correlated to urinary protein excretion levels, and DDAH overexpression but not Hyz prevented the increase in proteinuria. These findings were consistent with the previous report of Caglar et al. (27) that showed that ADMA levels were correlated with proteinuria in patients with CKD stage 1. Impaired NO production is the characteristic feature of endothelial dysfunction (1). There is a growing body of evidence that endothelial dysfunction is linked to proteinuria (43–45). Furthermore, ADMA levels are related to endothelial dysfunction in an animal model and patients with CKD (46,47). These observations suggest that the elevation of ADMA levels may exacerbate proteinuria in our CKD model by suppressing the generation of NO.

Conclusion

These data suggest that ADMA may be involved in peritubular capillary loss and tubulointerstitial fibrosis, thereby contributing to the progression of CKD. Although these results may be one of many mechanisms of how ADMA causes deterioration of renal function, substitution of DDAH protein or enhancement of its activity may become a novel therapeutic strategy for the treatment of CKD.

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Disclosures

None.

References


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See the related editorial, “A New Perspective for the Treatment of Renal Diseases?” on pages 1365–1367.