Involvement of asymmetric dimethylarginine (ADMA) in tubulointerstitial ischaemia in the early phase of diabetic nephropathy

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Abstract

Background. Decreased peritubular capillary (PTC) flow due to impaired endothelial function elicits tubulointerstitial ischaemia, thereby enhancing renal damage in chronic kidney disease, including diabetic nephropathy. Since nitric oxide (NO) is a vasodilator and known to play an important role in the maintenance of PTC flow, it is conceivable that asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, may cause tubulointerstitial ischaemia, thus being involved in the progression of diabetic nephropathy. In this study, we investigated whether overexpression of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that degrades ADMA, could improve tubulointerstitial ischaemia in streptozotocin (STZ)-induced diabetic rats.

Methods. Recombinant adenovirus vector encoding DDAH-I (Adv-DDAH) or control vector expressing bacterial β-galactosidase (Adv-LZ) was intravenously administrated to diabetic rats. Three days after the treatment, effects of DDAH overexpression on plasma or urinary levels of ADMA or NO metabolites (NOx), tubulointerstitial ischaemia and renal expression of transforming growth factor-β (TGF-β) were evaluated.

Results. Renal DDAH expression and activity were reduced in diabetic rats. Urinary levels of ADMA and TGF-β were increased, while NOx levels were decreased in diabetic rats. Compared with control rats, pimonidazole-detected hypoxic areas were larger in the kidney of diabetic rats, although the number of capillaries in tubulointerstitial regions was not different between the two groups. In addition, renal expression levels of hypoxia-inducible factor-1α (HIF-1α) and TGF-β were also increased in diabetic rats. DDAH overexpression significantly inhibited the increase of ADMA and the decrease of NOx and subsequently decreased urinary albumin excretion levels and ameliorated tubulointerstitial hypoxia and HIF-1α as well as TGF-β expression in diabetic rats.

Conclusion. The present study demonstrated for the first time that the suppression of ADMA by DDAH overexpression could improve tubulointerstitial ischaemia and subsequent renal damage in experimental diabetic nephropathy. Substitution of DDAH protein or enhancement of its activity may become a novel therapeutic strategy for the treatment of early diabetic nephropathy.

Keywords: asymmetric dimethylarginine; diabetic nephropathy; dimethylarginine dimethylaminohydrolase; endothelium; ischaemia

Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal failure, which could account for disabilities and high mortality rates in patients with diabetes [1]. DN is characterized by functional and structural changes in the glomerulus, such as glomerular hyperfiltration, thickening of glomerular basement membranes and an expansion of extracellular matrix in mesangial areas [1]. However, it has recently been recognized that proximal tubular atrophy and tubulointerstitial fibrosis are more important than glomerulosclerosis in terms of renal prognosis [2,3]. Furthermore, accumulating evidence suggests that chronic renal hypoxia may have an important role in the progression of tubulointerstitial fibrosis in chronic kidney disease (CKD) including DN [2–4]. Chronic renal hypoxia could be elicited by several factors such as loss of peritubular capillaries (PTCs), decreased PTC flow, decreased nitric oxide (NO) production and/or bioavailability and activation of the renin–angiotensin system [2,3]. Indeed, Kang et al. recently demonstrated that the inhibition of NO synthase (NOS) accelerated renal damage in a remnant kidney model by eliciting PTC loss [5,6]. Since NO is not only a vasodilator but also a mediator of angiogenic signal [7], it is conceivable that decreased NO production and/or bioavailability may be linked to PTC loss and/or impaired PTC flow, which could contribute to tubulointerstitial ischaemia and fibrosis in DN.

Increased levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, are associated with endothelial dysfunction in diabetes, which could
account for accelerated atherosclerosis in this population [8–13]. Further, recently, we have shown that the reduction of ADMA by overexpression of dimethylarginine dimethylaminohydrolase (DDAH), a rate-limiting enzyme that mainly degrades ADMA, inhibits the progressive loss of PTCs in remnant kidney model rats, thereby protecting against renal damage in a rat model of CKD [14]. These observations led us to speculate that increased ADMA level may be a causative factor of PTC loss or impaired PTC flow, which could cause tubulointerstitial ischaemia and fibrosis in DN. Therefore, in this study, we investigated whether overexpression of DDAH could improve tubulointerstitial ischaemia and damage via decreased ADMA levels in streptozotocin (STZ)-induced diabetic rats.

Methods

Animal preparation

Seven-week-old male Sprague-Dawley rats received 60 mg/kg intraperitoneal injection of STZ in a 10 mmol/L citrate buffer. Control non-diabetic rats (control: n = 10) received a citrate buffer alone. Animals with blood glucose levels >350 mg/dL 48 h later were considered to be diabetic. Fourteen days after the injection, rats were divided into two groups: diabetic rats treated with tail vein injection of 1.5 × 10^8 plaque-forming units of control vector expressing bacterial β-galactosidase (Adv-LZ) (STZ + Adv-LZ: n = 10) and those with that of recombinant adenovirus vector encoding DDAH-I (Adv-DDAH) (STZ + Adv-DDAH: n = 10) [14–16]. Three days after adenovirus infection, the rats were killed. As shown in the previous publications [14,15], we confirmed that adenoviral DDAH infection actually increased its expression in liver and kidney (Figure 1A).

Chemical analysis

Urinary albumin excretion (UAEx) levels were determined with commercially available ELISA kits (Exocell, Philadelphia, PA, USA). Plasma and urinary levels of NO metabolites (NOx: nitrate plus nitrite), L-arginine, ADMA and symmetric dimethylarginine (SDMA) were measured by a high-performance liquid chromatography as described previously [14–16].

Measurement of enzymatic activity of DDAH

Total DDAH activity was measured as described previously [14–16]. Briefly, homogenized kidney tissues were incubated with 4 μmol/L ADMA and 0.1 mol/L sodium phosphate buffers (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 an equal volume of 10% trichloroacetic acid, 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.

Immunohistochemistry

The kidneys were removed and fixed in 4% paraformaldehyde. Then the kidneys were embedded in paraffin wax for sectioning. Three-micrometre paraffin sections were incubated with a monoclonal JG-12 antibody raised against hypoxia-inducible factor-1α (HIF-1α) was purchased from Novus Biologicals (Littleton, CO, USA), and a polyclonal antibody directed against endothelial NOS (eNOS) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Western blot analysis

The kidney cortex tissues were homogenized and lysed with 25 mmol/L Tris–HCl (pH 7.4) containing 1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA and 1% protease inhibitor cocktail (Nakarai Tesque, Kyoto, Japan). Then the supernatant was separated by SDS–PAGE and transferred to nitrocellulose membranes (Biorad, Hercules, CA, USA) as described previously [18]. Immune complexes were visualized with an enhanced chemiluminescence detection system (ECL; Amersham Bioscience, Buckinghamshire, UK). A monoclonal antibody against hypoxia-inducible factor-1α (HIF-1α) was purchased from Novus Biologicals (Littleton, CO, USA), and a polyclonal antibody directed against endothelial NOS (eNOS) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Semi-quantitative reverse transcription-polymerase chain reactions (RT-PCR)

Poly(A)^+ RNAs were isolated from the kidney and then analysed by RT-PCR as described previously [14,15]. Forward and reverse primer sequences were 5'-CCTTGTGGCCTGTTGCGAGA-3' and 5'-CAGTTCAGACATGCTCACCGGGG-3' for detecting DDAH-I, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1, 5'-AGAATTGTGGAGATGGGGGAGA-3' and 5'-CAACCAGGACCGAGAAAGAGAC-3' for detecting DDAH-II, 5'-AACACTGAAGCTCGCACTCTCG-3' and 5'-TCAGCACAGATCTCTTGTGC-3' for detecting TGF-β1.
**Fig. 1.** Effects of diabetes or DDAH-I overexpression on DDAH-I, DDAH-II and PRMT1 expression in liver (A) and kidney (B). Upper panels (a) show the representative results of RT-PCR. Lower panels show the quantitative data of DDAH-I (b), DDAH-II (c) and PRMT1 (d) gene expression. Data were normalized by the intensity of GAPDH mRNA-derived signals and related to the value of the control. (e) Renal enzymatic activity of DDAH. ∗P < 0.05 compared to the value of the control. ∗∗P < 0.05 compared to the value of STZ-Adv-LZ.

### Statistical analyses

All data are presented as means ± SE. Analysis of variance (ANOVA) was performed for all studied parameters with Scheffe’s post hoc test to compare variables among experimental groups. A P-value <0.05 was considered statistically significant.

### Results

#### Clinical parameters of animals

As shown in Table 1, compared with the control, plasma glucose levels were elevated and body weights were significantly lower in STZ+Adv-LZ. Overexpression of DDAH did not affect glucose levels or body weight in diabetic rats. There were no significant differences of systolic blood pressure, heart rate or serum creatinine levels among the three groups. Creatinine clearance and urinary albumin excretion (UAE) levels were increased in diabetic rats (Table 1). DDAH overexpression significantly reduced UAE levels, but not creatinine clearance. Further, as shown in Figure 1B, renal DDAH-I gene expression and activity were decreased in diabetic rats, which were restored with the treatment of the adenoviral DDAH-I gene transfer. Renal and liver gene expressions of DDAH-II and protein arginine methyltransferase 1 (PRMT1), an important enzyme for ADMA synthesis, were not different among the three groups (Figure 1).

Plasma ADMA levels tended to increase in STZ+Adv-LZ, which was significantly reduced by DDAH infection (Figure 2A). Compared with non-diabetic control, urinary excretion levels of ADMA were increased and NOx levels were decreased in STZ + Adv-LZ, both of which were suppressed by DDAH overexpression (Figures 2C and 1F). There were no significant differences in plasma or urinary levels of SDMA, an inert isomer of ADMA, which is not degraded by DDAH (Figure 2B and D), among the groups. Further, plasma L-arginine levels were significantly lower in STZ+Adv-LZ, which were not affected by the treatment with DDAH infection (Figure 2E).

#### Measurement of the number of renal capillaries

We first examined the effects of diabetes or DDAH overexpression on PTC loss in our models. For this, renal capillary ECs were stained with a JG-12 antibody directed against
Table 1. Clinical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 10)</th>
<th>STZ + Adv-LZ (n = 10)</th>
<th>STZ + Adv-DDAH (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>152 ± 10</td>
<td>512 ± 23*</td>
<td>476 ± 34*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>370 ± 6</td>
<td>285 ± 7*</td>
<td>286 ± 8*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>106 ± 3</td>
<td>110 ± 3</td>
<td>113 ± 2</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>351 ± 8</td>
<td>302 ± 20</td>
<td>338 ± 21</td>
</tr>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>0.24 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>3.4 ± 0.08</td>
<td>4.2 ± 0.45*</td>
<td>3.7 ± 0.56</td>
</tr>
<tr>
<td>Urinary albumin excretion (mg/g creatinine)</td>
<td>28.0 ± 6.1</td>
<td>263 ± 56*</td>
<td>101 ± 35**</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the value of the control.
**P < 0.05 compared with the value of the Adv-LZ-treated diabetic rats.

Fig. 2. Plasma and urinary levels of methylated arginine, L-arginine and NOx. Plasma levels of ADMA (A), SDMA (B) and L-arginine (E) and urinary levels of ADMA (C), SDMA (D) and NOx (E) were measured by an HPLC. *P < 0.05 compared to the value of the control. **P < 0.05 compared to the value of the STZ + Adv-LZ.

aminopeptidase P, a specific marker for ECs [14]. As shown in Figure 3, there were no significant differences in the number of renal capillaries in tubulointerstitial or glomerular regions among the three groups. We also confirmed that expression levels of endothelial NOS, another marker for ECs, were not different among the groups by western blot analysis (data not shown).

Effects of DDAH overexpression on tubulointerstitial ischaemia

We next investigated the effects of diabetes or DDAH overexpression on tubulointerstitial ischaemia in our models. For this, we immunostained hypoxic areas by using pimonidazole, a hypoxic probe [3,4,17]. As shown in
Figure 4A and B, intensity of pimonidazole staining was increased in tubulointerstitial areas of the kidney cortex of STZ+Adv-LZ, which was significantly blocked by DDAH overexpression. Further, renal expression of HIF-1α protein was up-regulated in STZ+Adv-LZ, which was also suppressed by the treatment with Adv-DDAH (Figure 4C).

Effects of DDAH overexpression on TGF-β expression

Since hypoxia has been reported to stimulate TGF-β synthesis in tubular cells [12,20], we further studied the effects of DDAH overexpression on TGF-β expression in the kidney. As shown in Figure 5A, semi-quantitative RT-PCR revealed that DDAH overexpression inhibited up-regulation of renal TGF-β gene expression in STZ+Adv-LZ. Urinary excretion levels of TGF-β were increased in STZ+Adv-LZ, which were also blocked by the treatment with Adv-DDAH (Figure 5B).

Discussion

The salient finding of this study was that overexpression of DDAH, a rate-limiting enzyme that mainly degrades ADMA, not only decreased plasma and urinary excretion levels of ADMA, but also improved the increase in UAE and tubulointerstitial ischaemia and subsequently suppressed TGF-β up-regulation in the early phase of experimental DN.

There are several papers to show that plasma levels of ADMA are elevated in diabetic animals or patients, thus being involved in vascular injury and accelerated atherosclerosis in diabetes [8,9]. Therefore, the present study has extended these previous findings showing that elevation of ADMA levels may participate in tubulointerstitial ischaemia, thereby contributing to the development and progression of DN. In this study, renal DDAH-I expression and activity were decreased in diabetic rats, which were ameliorated by the treatment of the adenoviral DDAH-I gene transfer. Further, overexpression of DDAH not only lowered urinary levels of ADMA, as well as increased the reduced levels of urinary NOx generation, but also improved tubulointerstitial ischaemia in STZ+Adv-LZ. These observations suggest that the decreased metabolism of ADMA by DDAH may be mainly involved in tubulointerstitial ischaemia in DN. In support of this speculation, decreased enzymatic activity or expression of DDAH has been reported in STZ-induced diabetic rats, which could be correlated with the elevation of ADMA in this animal [8,21]. In the present study, we found that urinary excretion levels of
Fig. 4. Tubulointerstitial ischaemia. (A) Immunohistochemical staining of hypoxic area with pimonidazole. (a) and (d) Control; (b) and (e) STZ+Adv-LZ; (c) and (f) STZ+Adv-DDAH. (a)–(c) Magnification ×12.5; (d)–(f) magnification ×40. (B) Quantitative analysis of pimonidazole staining. *P < 0.01 compared to the value of the control. **P < 0.01 compared to the value of STZ-Adv-LZ. (C) Western blot analysis for HIF-1α. The upper panel shows the representative results of western blotting. The lower panel shows the quantitative data. Data were normalized by the intensity of β-actin and related to the value of the control (n = 10, each). *P < 0.05 compared to the value of the control.

Fig. 5. Effects of DDAH overexpression on TGF-β expression. (A) The upper panel shows the representative results of RT-PCR. The lower panel shows the quantitative representation of TGF-β gene induction. Data were normalized by the intensity of GAPDH mRNA-derived signals and related to the value of the control (n = 10, each). *P < 0.05 compared to the value of the control. (B) Urinary levels TGF-β (n = 10, each). *P < 0.05 compared to the value of the STZ-Adv-LZ.
SDMA, a structural isomer of ADMA, were not changed among the three groups (Figure 2D). Since cellular uptake of SDMA is mediated by a γ+ transporter, which is also known to be involved in ADMA uptake [22,23], it is unlikely that decreased tubular uptake could play a role in increased urinary excretion of ADMA.

As previously reported by other researchers [24,25], we found that L-arginine levels were decreased in diabetic rats. In this study, reduced urinary NOx generation and tubulointerstitial ischaemia were ameliorated by the treatment of Adv-DDAH, although DDAH infection did not affect the L-arginine levels in STZ+Adv-LZ. Further, it has been reported that L-arginine concentration as low as 3 μM is sufficient to induce half-maximal activity of NOS in vitro [26,27]. These observations suggest that it is unlikely that reduced L-arginine levels could contribute to the decrease in urinary NOx generation and tubulointerstitial ischaemia in our models. However, we cannot totally exclude the possibility that L-arginine could play a role in our systems because there are numerous studies that L-arginine supplementation could augment NO production in humans and thereby improve endothelium-dependent vasodilatation [28]. In vivo, specifically, in the presence of ADMA, L-arginine concentration as low as 3 μM may ‘NOT’ be sufficient to induce half-maximal activity of endothelial NOS, and therefore the L-arginine/ADMA ratio could be a good marker for NO generation [28,29].

NO is not only a vasodilator but also a mediator of the angiogenic signal [7]. Therefore, it is conceivable that elevation of ADMA could cause PTC loss and/or impaired PTC flow by reducing renal production of NO, which may in concert contribute to tubulointerstitial ischaemia and fibrosis in DN. In this study, diabetes or DDAH overexpression did not affect PTC loss (Figure 3). Therefore, decreased metabolism of ADMA by DDAH may cause tubulointerstitial ischaemia via impaired PTC flow in our models.

In the present study, UAE levels were increased in diabetic rats, which were blocked by the treatment of DDAH infection (Table 1). Caglar et al. previously reported that ADMA levels were correlated with proteinuria in patients with CKD stage I [30]. Since there is a growing body of evidence that endothelial dysfunction is linked to proteinuria [31–33], the present observations suggest that DDAH could ameliorate endothelial dysfunction and subsequently reduce UAE levels in diabetic rats via suppression of ADMA.

We have previously shown that DDAH overexpression down-regulates TGF-β expression in a rat remnant kidney model [14]. In the present study, TGF-β overexpression in the diabetic kidney was decreased by the treatment with Adv-DDAH. Several pieces of evidence have implicated the TGF-β as a major etiologic agent in the pathogenesis of tubulointerstitial fibrosis in DN [34,35]. Furthermore, there are several papers to show that the TGF-β gene is up-regulated under hypoxic conditions [20]. These observations suggest that the ADMA-mediated tubulointerstitial ischaemia may be involved in TGF-β induction and tubulointerstitial fibrosis in DN. In addition, exogenous administration of ADMA to humans caused a long-lasting decrease in renal perfusion even at doses that failed to alter blood pressure [36], thus further supporting the concept that ADMA could elicit tubulointerstitial ischaemia in the early phase of DN.

A number of studies about the effects of high glucose and/or diabetes on the renal NO system have often produced contradictory findings [37]. The use of different techniques for estimating the renal NO concentration and activity may explain some of the discrepancies. However, Keynan et al. reported that urinary NO production and renal NOS levels and activity determined by combination techniques, including immunoblotting, immunohistochemistry and diaphorase staining, were reduced during the early phase of experimental diabetes mellitus [38]. In addition, Palm et al. have recently shown that the reduced bioavailable NO concentration in the renal cortex directly measured by microsensors is associated with the decreased renal blood perfusion in the early phase of STZ-induced diabetes [25]. These findings support our concept that ADMA could contribute to early DN by causing tubulointerstitial ischaemia via suppression of renal NO generation.

In conclusion, the present observations suggest the active participation of ADMA–DDAH axis in tubulointerstitial ischaemia in DN. Recently, it has been reported that plasma levels of ADMA could be a strong predictor for the progression of renal dysfunction in patients with CKD [39,40], further supporting the clinical relevance of ADMA in chronic ischaemia and progression of renal injury. Substitution of DDAH protein or enhancement of its activity may become a novel therapeutic strategy for the treatment of DN. To show the direct evidence for the cause–effect relationship between NO bioavailability and tubulointerstitial ischaemia, whether NOS inhibition by l-NMMA could cause similar renal tubular damage in diabetic rats and if L-arginine could restore such damages should be clarified.

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Conflict of interest statement. None declared.

References


ADMA and renal ischaemia in diabetic nephropathy


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