Role of kidney ADP-riboyl cyclase in diabetic nephropathy

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Kim S, Park K, Gul R, Jang KY, Kim U. Role of kidney ADP-riboyl cyclase in diabetic nephropathy. Am J Physiol Renal Physiol 296: F291–F297, 2009. First published December 10, 2008; doi:10.1152/ajprenal.90381.2008.—The role of ADP-riboyl cyclases (ADPR-cyclases) in diabetic nephropathy was investigated. ADPR-cyclases synthesize cADP-ribose (cADPR), a Ca2+ mobilizing second messenger, and are stimulated by G protein-coupled receptors. We have previously reported that ADPR-cyclases can be activated by ANG II and showed that a specific kidney ADPR-cyclase inhibitor, 4,4'-dihydroxyazobenzene (DHAB), can protect ANG II-mediated mesangial cell growth (Kim SY, Gul R, Rah SY, Kim SH, Park SK, Im MJ, Kwon HJ, Kim UH. Am J Physiol Renal Physiol 294: F982–F989, 2008). In this study, we examined the preventive effect of DHAB on glomerular injury in streptozotocin (STZ)-induced diabetic mice. Male mice were randomly assigned to normal control and diabetic groups of comparable age. A diabetic group received 45 mg/kg of DHAB for 6 wk via daily intraperitoneal injections. Several nephropathy parameters were improved in the DHAB-treated diabetic group compared with the diabetic group, including urinary albumin (diabetic, 44.6±5.1 vs. treated, 33.9±3.9 μg/day), creatinine clearance (diabetic, 0.72±0.03 vs. treated, 0.83±0.04 ml/min 1.100 g−1), ratio of kidney to body weight (diabetic, 2.5±0.04 vs. treated, 1.4±0.04), and mesangial matrix expansion (diabetic, 13.9±2.2 vs. treated, 8.5±2.0%). These results indicate that kidney function in STZ-induced diabetes was improved by DHAB administration. Furthermore, DHAB inhibited phosphorylation of Akt and nuclear factor of activated T cell 3 nuclear translocation, as well as ADPR-cyclase activity and cADPR production, which were increased in the kidneys of the diabetic group. In addition, DHAB treatment decreased fibrosis marker protein expression and glomerular hypertrophy in the diabetic kidney. These findings indicate a crucial role that ADPR-cyclase signaling plays in the renal pathogenesis of diabetes and provide a therapeutic tool for the treatment of renal diseases.

angiotensin; cADP-ribose; 4,4'-dihydroxyazobenzene

ADP-riboyl cyclase(s) (ADPR-cyclase) are regulated through G protein-coupled receptor signaling, including the receptor for ANG II (9, 11, 16, 28). The product of ADPR-cyclase, cADP-ribose (cADPR), modulates Ca2+ fluxes in several types of cells (6, 9, 11). CD38, a representative ADPR-cyclase, has been known to be involved in signal transductions, such as cell growth, development of obesity, insulin secretion, and differentiation in mammalian cells (2, 15–17, 22). The existence of novel ADPR-cyclase(s) in the kidney, brain, or heart has been suggested from studies with CD38−/− mice (27). We recently demonstrated that ANG II-stimulated Ca2+ signals were not significantly different between CD38−/− and CD38+/+ cardiomyocytes but that 8-Br-cADPR completely inhibited the ANG II-induced sustained Ca2+ increase, indicating that cADPR is generated by a novel ADPR-cyclase (8). We also demonstrated that the kidney ADPR-cyclase-selective blocking compound 4,4'-dihydroxyazobenzene (DHAB) protects ANG II-mediated mesangial cell growth (16). In addition, we described underlying mechanisms of ANG II-mediated ADPR-cyclase activation in mouse mesangial cells: ANG II directly binds to AT1R, which sequentially activates phosphatidylinositol-3-kinase, protein tyrosine kinase, and phospholipase C-γ1, resulting in ADPR-cyclase activation and cADPR production (16). In this study, we investigated whether pharmacological inhibition of ADPR-cyclase can be effective in protection from diabetic nephropathy. To examine this hypothesis, we compared urinary albumin excretion, creatinine clearance level, phosphorylation of Akt, and nuclear factor of activated T cell (NFAT) nuclear translocation, histological change, and accumulation of extracellular matrix (ECM) protein in diabetic and DHAB-treated diabetic mice groups. Treatment with DHAB significantly protects the hypertrophic responses in diabetic mice. These results indicate that kidney ADPR-cyclase can be an important therapeutic target molecule in diabetic nephropathy.

MATERIALS AND METHODS

Reagents and antibodies. DHAB, streptozotocin (STZ), nicotinamide guanine dinucleotide (NGD•), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies were obtained from the following sources: anti-phospho-Akt (Ser473) monoclonal antibody (mAb), and anti-Akt polyclonal antibody (pAb) was from Cell Signaling Technology (Beverly, MA); anti-NFAT3 mAb, anti-fibronectin pAb, anti-collagen IV pAb, and anti-transforming growth factor (TGF)-β1 pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG, anti-goat IgG, and anti-rabbit IgG were purchased from Santa Cruz Biotechnology.

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Animals. C57Bl/6J male mice, 8 wk of age and free of murine-specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejon, Korea), housed throughout the experiments in a laminar flow cabinet, and maintained on standard laboratory chow ad libitum. All experimental animals used were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Standard guidelines for laboratory animal care were followed (14).

Treatment of STZ mice with DHAB. Male mice, weighing 20–25 g, were made diabetic by a single intravenous injection of STZ (75 mg/kg body wt) in 0.05 M citrate buffer (pH 4.8). On the same day, the control mice were injected with citrate buffer. After 2 days, induction of diabetes was confirmed by measurement of tail blood glucose level using a LifeScan One Touch glucometer (Johnson & Johnson). The diabetic mice (≥300 mg/dl blood glucose) were randomly divided into two groups; 6 mice/group were treated with vehicle (0.1% DMSO in saline, 100 μl) or DHAB (45 μg/kg body wt in 0.1% DMSO in saline, 100 μl) that was administered by intraperitoneal injection once a day for 6 wk. The control mice were divided into two groups; 6 mice/group were treated with the vehicle or DHAB. On day 39, the mice were detained in individual metabolic cages for 24 h for urine collection. On day 42, the mice were anesthetized with diethyl ether, and blood samples were taken from the abdominal aorta. Bilateral kidneys were rapidly removed and weighed. One kidney was immediately fixed in 10% formaldehyde in PBS, and the other kidney was frozen in liquid nitrogen and stored at −80°C.

Measurements of urinary albumin and creatinine. Urinary albumin and creatinine were measured at 6 wk from 24-h urine collection samples from mice housed in individual metabolic cages. During the urine collection, the mice were allowed free access to food and water. Albumin concentration in the urine was measured by a murine microalbuminuria kit (Albuwell, Exocell, Philadelphia, PA) according to the manufacturer’s protocols. Serum or urine creatinine level was measured by enzymatic method (SRL, Tokyo, Japan). Body weight-adjusted creatinine clearance was derived from the following formula: urinary creatinine × urine volume × 1.440 min⁻¹ × serum creatinine⁻¹ × body weight⁻¹ × 100 (33).

Measurements of plasma and kidney ANG II. ANG II levels in plasma and kidney were determined with a commercially available radioimmunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA) according to manufacturer’s instructions. In brief, blood was collected into chilled tubes containing anticoagulant. Blood samples were centrifuged at 4°C for 10 min at 1,000 g, and the supernatant was collected and stored at −80°C for further assay. The kidney was immersed in a fivefold volume of cold 1 M acetic acid and immediately homogenized. For the analysis of renal ANG II levels, kidney supernatants were dried. The dried residue was reconstituted in 4 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA. Plasma and kidney extracts were incubated with ANG II antiseraum and 125I-labeled ANG II for 15 min at 4°C and then centrifuged for 15 min at 3,500 g. The supernatant was discarded, and the radioactivity counts per minute (cpm) were recorded and then calculated by a computer.

Measurements of cADPR concentration and ADPR-cyclase activity. Mouse glomeruli were isolated from the kidney using the differential sieving method (21). The level of cADPR was measured using a cyclic enzymatic assay as described previously (7, 28). ADPR-cyclase activity was measured by using NGD⁺ as a substrate. Samples were incubated with NGD⁺ (200 μM) in 0.1 M sodium phosphate buffer (pH 7.2) for 10 min. Fluorescence of cGDPR in the solution was determined at excitation/emission wavelengths of 297/410 nm (Hitachi F-2000 fluorescence spectrophotometer).

Immunoblotting. Protein extraction and immunoblotting of isolated glomeruli were performed as previously described (28). Cytosolic and nuclear fractions were prepared with a Pierce NE-PER nuclear and cytoplasmatic extraction kit according to the manufacturer’s directions (Pierce, Rockford, IL). Proteins (20 μg/lane) were resolved on 10 or 12% SDS-PAGE gel and transferred to polyvinylidene difluoride (GE Healthcare, Little Chalfont, Buckinghamshire, UK) membranes. Non-specific sites were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the membranes were then incubated overnight with primary antibodies (p-Akt and Akt, 1:2,500 dilution; fibronectin, collagen IV, TGF-β1, and NFAT3, 1:1,000 dilution) at 4°C. The blots were rinsed four times with TTBS (Tris-buffered saline with Tween 20) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilutions of each antibody) for 1 h at room temperature. The binding of the antibodies was visualized using an enhanced chemiluminescence system (Bio-Rad, Munich, Germany). Protein concentration was determined using a Bio-Rad protein assay kit, and known concentrations of BSA were used as the standard.

Histological analysis. Formalin-fixed kidney tissues were decalcified in EDTA for 5–7 days, dehydrated, and embedded in paraffin. Serial sections (4 μm) were stained with periodic acid-Schiff (PAS) reagents for light microscopic observation to determine glomerular volume and mesangial matrix index. The surface area (μm²) of 25 glomerular sections from each animal and PAS-positive area were quantified in digital images using a computer-assisted color image analyzer (LUZEX F, Nikon, Tokyo, Japan). The mesangial matrix fraction was determined as the percentage of mesangial PAS-positive area per total glomerular surface area.

Immunohistochemistry. Tissue sections (4 μm) were deparaffinized and rehydrated. Antigen retrieval was performed by incubation in target retrieval solution (DakoCytomation, Carpinteria, CA) at 95°C for 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min, and slides were rinsed with TTBS. Sections were blocked with the appropriate preimmune serum and then incubated with avidin/biotin blocking solutions (DakoCytomation). Slides were then incubated overnight at 4°C with a primary antibody for TGF-β1 (1:50 dilution), collagen IV (1:100 dilution), and fibronectin (1:100 dilution). The stained sections were then incubated with biotinylated anti-goat IgG or biotinylated anti-rabbit IgG (Sigma-Aldrich), and the
sections were incubated with horseradish peroxidase-conjugated streptavidin (DakoCytomation). The immune complexes were detected using a 3-amino-9-ethyl-carbazole solution (DakoCytomation) according to the manufacturer’s instructions. The sections were finally counterstained with hematoxylin solution (DakoCytomation) before being mounted.

Statistical analysis. Data represent means ± SE of at least three separate experiments. Statistical comparisons were performed using one-way ANOVA followed by Scheffe’s test. The statistical significance of difference between groups was determined using Student’s t-test. The effect of DHAB treatment on the change from baseline of the control mice group in body weight, kidney weight, urine albumin excretion, creatinine clearance, and blood glucose was compared with that of vehicle-treated group (control). *P < 0.01 vs. vehicle-treated group (control). #P < 0.01 vs. STZ group.

RESULTS

Effects of DHAB on renal function of STZ-induced diabetic mice. We utilized STZ to induce a mouse model of experimental DN. Administration of STZ to mice significantly increased the levels of blood glucose, the ratio of kidney weight to body weight, the level of blood glucose, and renal tissue (D). Values are means ± SE. *P < 0.01 vs. vehicle-treated group (control). #P < 0.01 vs. STZ group.

Effect of DHAB on ADPR-cyclase activity and levels of cADPR or plasma and kidney ANG II. We next examined whether DHAB could influence the plasma and kidney ANG II level in diabetic mice, since a previous study demonstrated a significant increase in angiotensinogen and renin mRNA expressions in the kidney of STZ-diabetic rats (1). In the kidney glomeruli, DHAB treatment significantly ameliorated the increased ADPR-cyclase activity and cADPR production in the hyperglycemic mice group (Fig. 2, A and B). Figure 2, C and D, shows that the plasma and kidney ANG II levels were greatly increased in mice treated with STZ alone or STZ plus DHAB compared with those in vehicle or DHAB control mice, indicating that DHAB had no effect on the increase in the ANG II level in diabetic mice.

DHAB inhibits Akt phosphorylation and nuclear translocation of NFAT3 in the diabetic kidney. We previously showed that activated ADPR-cyclase by ANG II stimulated the phosphorylation of Akt and NFAT3 nuclear translocation (16). Thus we next studied whether DHAB treatment could affect the level of phospho-Akt or NFAT3 nuclear translocation in the diabetic kidney. As shown in Fig. 3, A and B, the level of phospho-Akt was greatly increased in the kidney of diabetic mice, accompanied by no change of Akt protein level. Treatment with DHAB significantly reduced the increased level of phospho-Akt. DHAB alone slightly lowered the phospho-Akt level, but a statistical significance was not found. NFAT proteins are present in the cytoplasm in the resting state, translocate into the nucleus after activation, and can activate target gene expression. This process has been shown to be regulated by dephosphorylation of the regulatory domain of NFAT by calcineurin (30). As shown in Fig. 3, C and D, induction of diabetes with STZ also increased the NFAT3 level.
in the nuclear fraction of the kidney, whereas the NFAT3 level in the cytosol was significantly reduced compared with that in the kidneys of the vehicle or DHAB control mice. Treatment with DHAB, however, markedly reduced the NFAT3 level in the nuclear fraction, while increasing the NFAT3 level in the cytosol fraction. DHAB alone did not show any effects on NFAT translocation, which was similar to that in the kidney of the vehicle control mice.

DHAB ameliorates glomerular injury induced by diabetes. In view of the impressive reduction of the kidney weight-to-body weight ratio and albuminuria in DHAB-treated diabetic mice (Fig. 1, B and D), we assessed renal histology by staining with PAS reagents. Histological data revealed that diabetic mice showed significant glomerular hypertrophy, slight segmental increments in mesangial, and slight and focally distributed mesangial cell proliferation compared with those in the vehicle or DHAB control mice. Treatment of diabetic mice with DHAB ameliorated the above pathogenic findings (Fig. 4, A and B). The glomeruli of diabetic mice showed an increase in the accumulation of PAS-positive matrix in the mesangium compared with those of control mice. Diabetic mice treated with DHAB showed relatively mild mesangial matrix expansion compared with diabetic mice (Fig. 4C). In control mice, the mesangial matrix expansion of the total glomerular tuft area was 5.5 ± 1.5%, but that was increased significantly to 13.9 ± 2.2% in diabetic mice. DHAB treatment remarkably decreased the mesangial matrix fraction to 8.5 ± 2.0%, DHAB itself having no effect on normal mice (6.3 ± 1.2%) (Fig. 4C), suggesting that the inhibition of ADPR-cyclase activation contributed to blocking of renal pathogenesis in diabetic mice.

DHAB reduces ECM accumulation in the kidney of diabetic mice. One of the important factors in kidney failure is fibrosis (23, 35). Mesangial matrix expansion is the most prominent pathological feature of DN, which is characterized by the accumulation of ECM proteins, such as collagen IV and fibronectin, in the glomeruli (4, 12). Therefore, we examined the expression of representative fibrosis marker proteins, fibronectin and collagen IV, along with TGF-β, which is known to stimulate transcription of ECM genes in renal cells (4, 12, 35). Figure 5A illustrates the deposition of TGF-β1, fibronec-

![Fig. 4. Light microscopic appearance of glomeruli. A: representative photomicrographs of the kidney sections stained with periodic acid-Schiff (PAS). Scale bars = 50 μm. B: quantification of glomerular size in A. MAG; mean area of glomeruli. Glomerular cross-sectional areas were determined by using a computer-assisted color image analyzer. C: quantification of extracellular mesangial matrix expansion is expressed as PAS-positive mesangial material per total glomerular tuft cross-sectional area (mesangial area/total glomerular tuft area × 100). Values are means ± SE from 25 individual glomeruli in kidney sections from 6 mice in each group. *P < 0.05 vs. control. #P < 0.05 vs. STZ.](http://ajprenal.physiology.org/)

![DHAB ameliorates glomerular injury induced by diabetes.](http://ajprenal.physiology.org/)
tin, and type IV collagen in renal glomeruli in the diabetic kidney. To determine the effect of DHAB on ECM deposition in glomeruli, we analyzed the TGF-β1, fibronectin, and type IV collagen expression in the isolated glomeruli. As presented in Fig. 5B, D, and F, Western blot analysis demonstrated that DHAB treatment suppressed the accumulation of each ECM protein in the glomeruli of diabetic kidneys. These results demonstrate that ADPR-cyclase activation in diabetes is associated with diabetic renal fibrosis, which is avertable with DHAB treatment.

DISCUSSION

The biochemical properties of kidney ADPR-cyclase are different from those of the prototype ADPR-cyclase, CD38 (25, 27). We recently observed that DHAB was a potent inhibitor of kidney ADPR-cyclase, which made it possible to elucidate involvement of ADPR-cyclase/cADPR in ANG II signaling in the kidney (16, 25). In this study, we investigated the effects of DHAB on a mouse model of STZ-induced diabetes and demonstrated that DHAB treatment significantly ameliorated albuminuria and downregulated the expression of fibrogenic factor TGF-β1, subsequently reducing mesangial matrix protein production in diabetic mice kidney, without, however, changing serum glucose levels. ADPR-cyclase was significantly activated, and the cADPR level was also increased in the diabetic kidney, and they were recovered by DHAB treatment. On the other hand, the plasma and kidney ANG II level was elevated in both the diabetic and DHAB-treated diabetic mice group. This result suggests that DHAB affects only ADPR-cyclase activation, but not the plasma and kidney ANG II level in the diabetic experimental model. Furthermore, consistent with a previous study (16), DHAB suppressed also the levels of phospho-Akt and NFAT3 nuclear translocation in diabetic kidneys.

NFATs were first found in T lymphocytes as transcription factors (26, 30). Although the distribution of NFAT was originally thought to be restricted to lymphoid systems, the expression of NFATs has been shown to be ubiquitous (13, 29). In cardiomyocytes, NFAT3 has been found to induce expression of brain natriuretic peptide, a peptide overexpressed in the hypertrophic heart, through its binding to the promoter region of the brain natriuretic peptide gene (24), and it can be regulated by ADPR-cyclase, in turn participating in mesangial cell proliferation/hypertrophy (16). In the present study, we demonstrated that DHAB, a ADPR-cyclase inhibitor, prevented the increased nuclear translocation of NFAT3 in STZ-induced diabetic kidneys.

Even though different mechanisms are involved, alterations of mesangial cell proliferation and ECM production are believed to play predominant roles in the pathogenesis of progressive glomerulosclerosis which leads to ESRD (20, 31). In the process of tissue development and wound healing, TGF-β1 plays a crucial role in controlling the ECM deposition and remodeling; TGF-β1 stimulates the synthesis of major components of ECM proteins, such as collagen and fibronectin (4, 23, 35). In diabetic kidneys, the overexpression of TGF-β1 is believed to be the major mediator responsible for the early pathological changes of DN, including the glomerular basement membrane thickening and mesangial matrix expansion (20, 23). The present study revealed that DHAB treatment effectively ameliorated the increased expression of TGF-β1 as well as fibronectin and collagen IV in diabetic kidneys. Histor-
logical analysis also showed that DHAB significantly reduced glomerular hypertrophy in the kidney of diabetic mice.

DHAB is a bisphenol analog. Polyphenolic compounds have been widely used to exploit antioxidant effects in a wide range of diseases. Tannic acid, a plant polyphenol, has been demonstrated to reduce ROS production by modulating enzymes involved in oxidative metabolism in the mouse kidney (18). Tea polyphenols were used to effectively counteract the apoptosis of tubular and interstitial cells in rats with cyclosporine-induced chronic nephrotoxicity (32). We have tested the antioxidant effect of DHAB on ANG II-induced ROS generation in mesangial cells and found that DHAB did not block the ANG II-induced ROS generation with a similar concentration that inhibits ADPR-cyclase in mesangial cells (data not shown). Therefore, these findings rule out the possibility of the action of DHAB as an antioxidant in our present studies.

In conclusion, our study is the first to demonstrate that increased activity of ADPR-cyclases played a critical role in experimentally induced DN. DHAB treatment showed an antihypertrophic effect on diabetic kidneys and prevented nephropathy, independent of blood glucose as well as ANG II levels. These findings suggest that ADPR-cyclase is associated with the pathogenesis of DN and can be a therapeutic target in DN.

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