Angiotensin 1–7 mediates renoprotection against diabetic nephropathy by reducing oxidative stress, inflammation, and lipotoxicity

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The prevalence of diabetes mellitus (DM) is rapidly increasing worldwide driven by the rise in the prevalence of obesity and type 2 DM (51). Hyperglycemia due to insulin resistance is regarded as a central and pathogenic feature of type 2 DM, contributing to the development of macro- and microvascular complications in diabetic patients, including diabetic nephropathy. Diabetic nephropathy is a most common cause of end-stage renal disease, but the underlying mechanism mediating diabetic nephropathy is incompletely understood (10, 23).

Several factors, including insulin resistance, renal lipid accumulation, inflammation, and activation of the renin-angiotensin system (RAS), are involved in the pathogenesis of diabetic nephropathy (2, 9). Adipose tissue is a key organ in insulin resistance, as adipocytes are the origin of an inflammatory process characterized by infiltration of macrophages and increased inflammatory cytokines, leading to the initiation of diabetic nephropathy (44). The RAS is closely associated with the generation of reactive oxygen species (ROS) in diabetic nephropathy (4, 9) while the prolonged hyperglycemia causes mitochondrial ROS production, leading to diabetic microvascular complications (16).

Blockade of the RAS is currently employed as a first-line treatment for diabetic nephropathy, and ANG II type 1 receptor (AT1R) antagonists can slow the progression of diabetic nephropathy (6, 39). Angiotensin-convertase enzyme 2 (ACE2), which cleaves ANG II into ANG 1–7, counterregulates the ACE/ANG II/AT1R axis (11, 14, 34, 43, 47). While genetic deletion of the Mas receptor leads to metabolic syndrome and renal injury (36), ANG 1–7 activates signaling pathways in renal triacylglycerol (TAG) levels in association with altered renal function (30, 31). Animal use in this study, we assessed the effects of ANG 1–7 treatment on diabetic nephropathy in db/db mice. ANG 1–7 treatment improved glomerular expansion, normalized albumin excretion, and reduced ROS production and inflammation and renal triacylglycerol (TAG) levels in association with altered signaling pathways. These results indicate that ANG 1–7 ameliorates diabetic nephropathy by decreasing lipotoxicity, oxidative stress, and inflammation and provides important insight into a potential new therapy for diabetic nephropathy.

MATERIALS AND METHODS

Experimental animals and protocol. Male C57BL/6J-lepr/lcpr (db/db) and C57BL/6J (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The db/db mouse is an established mouse model of type 2 DM and diabetic nephropathy (42). ANG 1–7 (0.5 mg·kg⁻¹·day⁻¹) or saline were administered for 28 days by micro-osmotic pumps (model 1002, Alzet, Cupertino, CA), which were implanted subcutaneously at the dorsum of the neck in 5-mo-old male db/db mice, as previously described (30, 31). Animal use in this investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and to the guidelines of the Canadian Council on Animal Care. All studies were approved by
the University of Alberta Health Sciences Animal Care and Use Committee.

Western blot analysis. Western blot analyses were performed, as reported previously (30, 31). The protein was extracted from kidney tissues by using homogenate buffer consisting of 50 mM Tris·HCl (pH 8 at 4°C), 1 mM EDTA, 10% glycerol (wt/vol), 0.02% Brij-35 (wt/vol), 1 mM dithiothreitol, and 1:1,000 protease inhibitors and 1:100 phosphatase inhibitors (Sigma). The protein was quantified with a Bradford protein assay kit (Bio-Rad). Protein samples were electrophoresed onto 8–10% SDS-polyacrylamide gels and transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). The membranes were incubated for blocking with 5% milk in Tris-buffered saline-Tween-20 (TBST) for 1 h and probed with either anti-sirtuin 1 (SIRT1; Cell Signaling), anti-adipose triglyceride lipase (ATGL; Cell Signaling), anti-peroxisome proliferator-activated receptor-α (PPARα; Abcam), anti-phospho signal transducer and activator of transcription 3 (STAT3; Cell Signaling), anti-total STAT3 (Cell Signaling), anti-phospho Erk1/2 (Cell Signaling), anti-total Erk1/2 (Cell Signaling), anti-ACE2 (Abcam), anti-α-tubulin (Cell Signaling), or anti-β-actin (Santa Cruz Biotechnology) antibodies. After extensive washing with TBST, immunoblots were visualized with an enhanced chemiluminescence Western blot detection kit (PerkinElmer) and quantified with Image J software (NIH, Bethesda, MD).

Serum creatinine and urinary albumin levels. The serum creatinine concentration was determined with a Mouse Creatinine Kit (Crystal Chem) as per the manufacturer’s instructions. The creatinine concentration from samples was interpolated using a standard calibration curve. Urine was collected from conscious mice, and the creatinine

Fig. 1. Angiotensin 1–7 (ANG 1–7) decreased kidney weight and reduced glomerular expansion and urinary albumin excretion in db/db mice. Kidney weight (A) and kidney weight corrected for tibial length (TL; B) were significantly decreased in response to ANG 1–7 despite the lack of change in the increased body weight (C). Periodic acid-Schiff staining showed ANG 1–7 reduced glomerular expansion (D and E). Serum creatinine was unchanged (F), while the increased albumin urinary excretion was completely normalized by ANG 1–7 in db/db mice (G). WT, wild-type; A.U., arbitrary units. Values are means ± SE of n = 8/group. *P < 0.05 compared with WT. †P < 0.05 compared with placebo-treated db/db group.
concentration in urine was measured by the Mouse Creatinine Kit (Crystal Chem). Urine creatinine was adjusted to 20 mg/dl with distilled water. The equivalent of 1 µl of urine was subjected to 12% SDS-PAGE, and a goat anti-mouse albumin primary antibody (catalog no: A90–134A, Bethyl Laboratories) and donkey anti-goat horseradish peroxidase conjugate (catalog no. sc-2020, Santa Cruz Biotechnology) were used for hybridization. Urinary albumin was quantified by densitometry of the Western blots.

**Tissue and plasma TAG and blood glucose measurements.** Tissue TAG was extracted from kidney tissue, and the samples were then dried under nitrogen gas at 60°C. Dried samples were dissolved in 50 µl of a 3:2 tert-butyl alcohol-Triton X-100-methyl alcohol mixture.

Fig. 2. ANG 1–7 treatment increased renal ANG 1–7 levels and decreased renal fibrosis with dephosphorylation of the signal transducer and activator of transcription 3 (STAT3) signaling pathway. Renal ANG II (A) and ANG 1–7 (B) levels in response to systemic ANG 1–7 treatment in db/db mice show a selective increase in renal ANG 1–7 levels. Masson’s trichrome (C), picrosirius red staining (D), and collagen volume quantification (CVF; E) showed increased renal fibrosis in db/db kidneys, which was markedly reduced by ANG 1–7. Phosphorylation of renal STAT3 (F) and Erk1/2 (G) pathways was significantly increased in db/db mice, while ANG 1–7 resulted in reduced phospho-STAT3 without affecting phospho-Erk1/2 levels. Values are the means ± SE of n = 5/group except in A and B, where n = 9 for WT and n = 10 for the db/db groups. *P < 0.05 compared with WT. †P < 0.05 compared with placebo-treated db/db group.
TAG levels were quantitated colorimetrically as glycerol using an enzymatic assay (Wako Pure Chemical Industries, Osaka, Japan) (32). Plasma TAG levels were also quantified colorimetrically using the same enzymatic assay. Blood glucose was measured using an Accu-Chek Aviva glucometer (Roche Diagnostics, Mannheim, Germany) (32).

Histology. Picro-sirius red (PSR), dihydroethidium (DHE), ACE2, oil-O red, and hematoxylin and eosin (H&E) staining were performed as previously reported (31, 35, 50). Images for PSR, DHE, and ACE2 staining were captured by fluorescence microscopy and analyzed using MetaMorph software (Olympus IX81, Center Valley, PA). F4/80 staining was performed in the adipose tissues as the cell surface marker of macrophages. Briefly, 5-μm thick sections were stained with anti-F4/80 (AbD Serotec) primary antibody and ImmPRESS reagent anti-rat Ig-G. ImmPACT AEC red was used as a substrate to develop red color for F4/80-positive areas, and the sections were also counterstained with hematoxylin to stain the nucleic acids. Oil-O red staining, F4/80 macrophages, and H&E staining were captured by light microscope (DM4000B, Leica). Autofluorescence imaging of adipocytes was used to assess the adipocyte cell size by fluorescence microscopy.

Renal ANG 1–7 and ANG II levels. Renal ANG 1–7 and ANG II levels were measured by radioimmunoassay in the Hypertension and Vascular Disease Centre Core Laboratory at Wake Forest University School of Medicine as previously described (50).

Renal and urinary ACE2 activity assay. Renal and urinary ACE2 activity was assessed using a fluorescent assay protocol as described previously (35, 48). Briefly, 10 μl of urine or 10 μg total protein was added to the black microtiter plate containing assay buffer with 20 μM 7-methoxycoumarin-YVADAPK-(2,4-dinitrophenyl)-OH (R&D Systems) as the fluorogenic substrate. The total volume was kept to 100 μl, and the assay was performed in
duplicates. DX-600 (Phoenix Pharmaceuticals) was used as a specific ACE2 inhibitor.

NADPH oxidase activity assay. We measured NADPH oxidase activity by using a lucigenin-enhanced chemiluminescence assay as reported previously (35, 50).

Statistical analysis. All data are presented as means ± SE. Statistical analysis of the data was performed using a Student’s t-test or one-way ANOVA followed by multiple comparison testing using Student-Neuman-Keuls (SNK) testing as appropriate using SPSS Statistics 19 software.

RESULTS

ANG 1–7 ameliorates mesangial expansion and renal fibrosis in diabetic nephropathy. Kidney weights in 6-mo-old male db/db mice were increased compared with WT mice (Fig. 1, A and B). ANG 1–7 treatment significantly decreased kidney weight in db/db mice (Fig. 1, A and B) without affecting the increased body weight (Fig. 1C). We performed periodic acid-Schiff (PAS) staining to assess the effect of ANG 1–7 treatment on mesangial expansion, a characteristic feature of diabetic nephropathy (26). PAS staining clearly showed that db/db mice had mesangial expansion, and ANG 1–7 treatment significantly reduced the size of the mesangium (Fig. 1, D and E). While serum creatinine was not elevated in db/db mice, urinary albumin excretion was increased and completely normalized by ANG 1–7 (Fig. 1, F and G).

Next, we measured renal levels of ANG II and ANG 1–7. While ANG 1–7 treatment did not alter renal ANG II levels (Fig. 2A), renal ANG 1–7 was significantly increased in the

Fig. 4. ANG 1–7 treatment reduced inflammation and adverse remodeling in perirenal adipose tissue. Hematoxylin and eosin (A) and F4/80 macrophage (B) staining and quantification (C) showed increased inflammation due to a primary increase in macrophages in the perirenal adipose tissue from db/db mice, which was reduced by ANG 1–7 treatment (A–C). Adipocyte cross-sectional area shown as autofluorescence images (D) and quantification (E) with assessment of interstitial fibrosis as determined by Masson’s trichrome staining (F) was increased in perirenal adipose tissue from db/db mice and reduced by ANG 1–7 treatment (D–F). Values are the means ± SE of n = 4/group. *P < 0.05 compared with WT.
db/db kidneys (Fig. 2B), confirming enhanced ANG 1–7 effects in the renal tissue. The diabetic milieu increases the activity of profibrotic and proinflammatory growth factors and cytokines, which increases renal fibrosis. Masson’s trichrome and PSR staining showed increased renal fibrosis in db/db mice while ANG 1–7 treatment significantly reduced renal fibrosis (Fig. 2, C–E). We next assessed the potential mechanism of pathological signaling pathways involved in diabetic nephropathy and fibrosis (1, 3, 5, 22). STAT3 activation is involved in the development of renal fibrosis (1, 5). In line with these studies, the phosphorylation of STAT3 was increased in the kidneys from db/db mice, and ANG 1–7 treatment decreased the phosphorylation of STAT3 (Fig. 2F). In contrast, the increased global phosphorylation of renal Erk1/2 was not reversed by ANG 1–7 treatment (Fig. 2G).

ANG 1–7 counteracts ROS production and reduces inflammation in perirenal adipose tissue. Mesangial matrix expansion is related to ROS-mediated injury (27). Hyperglycemia is also known to induce ROS production in the kidney, and an activated RAS produces ROS via ANG II-mediated NADPH oxidase activation (9, 19). Therefore, we assessed whether ANG 1–7 treatment inhibits renal ROS production in the kidney, and an activated RAS produces ROS via ANG II-mediated NADPH oxidase activation (9, 19). Thereafter, we assessed whether ANG 1–7 treatment inhibits renal ROS production in db/db mice. NADPH oxidase activity was increased in db/db kidneys (Fig. 3A). In this line with the result, the glomerular superoxide level was elevated (Fig. 3, B and C), resulting in increased levels of nitrotyrosine (Fig. 3D) in db/db kidneys. ANG 1–7 treatment lowered ROS production in db/db kidneys, in association with reduced NADPH oxidase activity and nitrotyrosine levels (Fig. 3, A–D). ANG 1–7-induced attenuation of ROS coupled with the insulin-sensitizing property of ANG 1–7 (12, 17) reduced the elevated random blood glucose (Fig. 3E) and plasma TAG (Fig. 3F) levels in the db/db mice.

These results confirm that ANG 1–7 treatment effectively attenuated ROS production in the kidneys from db/db mice. Inflammation is also a key driver of diabetic nephropathy, and inflammation in adjacent perirenal adipose tissue exacerbates diabetic nephropathy (45). Adipose tissue shows increased adipocyte size, macrophage infiltration, and fibrosis in the setting of metabolic derangements (46, 49). The perirenal adipose tissue from db/db mice showed a marked increase in inflammatory cells, concomitant with the increased adipocyte cross-sectional area and enhanced fibrosis in perirenal adipose tissue (Fig. 4, A–F). ANG 1–7 treatment resulted in reduced inflammation and decrement in adipocyte size, and ameliorated fibrosis in perirenal adipose tissue (Fig. 4, A–F).

ANG 1–7 treatment reversed renal lipotoxicity and upregulation of ACE2 levels. Lipotoxicity is a critical contributor to the progression of diabetic- and obesity-related nephropathy (33). Biochemical analysis of the kidney revealed the accumulation of TAG in the kidney from db/db mice, which was prevented by ANG 1–7 treatment (Fig. 5, A and B). We determined ATGL expression, which is a key mediator of TAG degradation. ATGL levels were decreased in db/db kidneys, and ANG 1–7 treatment upregulated ATGL expressions (Fig. 5C). Furthermore, we explored the

Fig. 5. ANG 1–7 treatment decreased accumulation of lipids and restored altered signaling in kidneys. Oil-O red staining showed a marked decrease in renal lipid accumulation in db/db mice in response to ANG 1–7 (A) while renal TAG (B) levels were significantly increased in db/db mice, accompanied by a decreased adipose triglyceride lipase (ATGL) level (C), which were all normalized by ANG 1–7 (A–C). Western blot analysis and quantification showed increased acetylation of FOXO1 in db/db kidneys (D), accompanied by reduced sirtuin 1 (SIRT1; E) and peroxisome proliferator-activated receptor-α (PPARα; F) levels, which were completely reversed by ANG 1–7 treatment. Values are the means ± SE of n = 5/group. *P < 0.05 compared with WT.
mechanism of ANG 1–7-induced upregulation of ATGL. FOXO1 is a key transcriptional factor involved in regulating energy metabolism and controls ATGL expression due to SIRT1-induced acetylation. Acetylation of FOXO1 was significantly increased in the kidneys from db/db mice, and ANG 1–7 treatment deacetylated FOXO1 (Fig. 5D). Consistent with these changes, SIRT1 expression was significantly reduced in db/db kidneys (Fig. 5E) combined with reduced PPARα expression (Fig. 5F), a key downstream target of SIRT1. ANG 1–7 treatment increased the expression of both SIRT1 and PPARα in the db/db kidneys (Fig. 5, E and F). These data suggest that ANG 1–7 induces renal ATGL expressions possibly via deacetylation of FOXO1 by SIRT1. We next determined renal ACE2 level in db/db mice since ACE2 is regulated by the SIRT1 pathway and in diabetic conditions (8, 35). Immunofluorescence imaging showed increased ACE2 levels in renal cortical tubules in db/db mice, which was reversed by ANG 1–7 treatment (Fig. 6A) and which was also confirmed by Western blot analysis (Fig. 6B). Assessment of renal ACE2 activity (Fig. 6C) revealed concordant changes compared with ACE2 protein (Fig. 6, A and B), which was closely mirrored by the alterations in urinary ACE2 activity (Fig. 6D).

**DISCUSSION**

The prevalence of obesity and type 2 DM has been increasing worldwide over recent decades. Macro- and microvascular...
complications are the major causes of morbidity and mortality in patients with DM, and diabetic nephropathy is now the leading cause of end-stage renal disease (10, 23). Consequently, new therapeutic strategies against diabetic nephropathy are needed. To assess the effect of ANG 1–7 on diabetic nephropathy, we randomized 5-mo-old db/db mice, a well-established type 2 DM model with diabetic nephropathy (7), to receive a placebo or ANG 1–7. We showed that 1) ANG 1–7 ameliorates diabetic nephropathy, reduces fibrosis and inflammation in the kidney, as well as perirenal adipose tissue; 2) ANG 1–7 attenuates renal ROS production via reduced NADPH oxidase activity; and 3) ANG 1–7 reduces renal lipotoxicity due to regulation of the SIRT1-FOXO1 pathway and increased ATGL levels.

Several factors contribute to the initiation and progression of diabetic nephropathy (9, 21). Inflammation in obese adipose tissue contributes to the development and progression of systemic insulin resistance and DM (13). Inflammatory cytokines involved in the progression of diabetic nephropathy can be derived from adipose tissue. Perirenal visceral adipose tissue in db/db mice showed increased inflammation, which was reduced by ANG 1–7 treatment. ROS are also critical factors in the progression of diabetic nephropathy, and chronic inhibition of NADPH oxidase eliminates podocyte apoptosis and mesangial expansion in diabetic kidneys (25, 41). A high-fat diet induces renal oxidative stress despite the adaptation of mitochondrial bioenergetics (40), suggesting that lipotoxicity is a key driver of ROS production. Renal fibrosis is a central and definitive event in the progression of chronic kidney disease. STAT3 can be activated by multiple cytokines and growth factors and plays a critical role in renal injury and fibrosis (1, 5, 22). We found that STAT3 is phosphorylated in the kidneys from db/db mice. ANG 1–7 treatment prevented the phosphorylation of STAT3, which can mediate renoprotective effects in a diabetic setting.

The ACE2/ANG 1–7 axis has emerged as a key determinant of diabetic nephropathy (34, 47). Urinary ACE2 levels are increased in db/db mice (48) as well as in humans with chronic kidney disease and DM (28). Thus urinary ACE2 has been proposed as a potential biomarker of kidney disease. Increased urinary ACE2 originates from enhanced renal tubular ACE2 protein due to shedding from the membrane of tubular epithelial cells. Increased renal ACE2 expression may have a beneficial effect and be renoprotective, since ACE2 counteracts the detrimental effects of ANG II (43, 50) and recombinant ACE2 ameliorates diabetic nephropathy (34). Indeed, renal ACE2 protein is increased predominantly in the renal tubules in the db/db mice (48), suggesting it seems to be enhanced to counteract RAS activation. ANG 1–7 treatment normalized renal ACE2 levels in db/db mice with corresponding changes in urinary ACE2 activity. Our systemic ANG 1–7 treatment did increase renal ANG 1–7 levels. However, renal ANG II and ANG 1–7 levels in db/db kidneys did not change compared with wild-type kidneys despite variation in renal ACE2 levels and activity, suggesting alternative enzymatic pathways for processing angiotensin peptides in the kidneys, such as nephrilysin and prolyl carboxypeptidase (18, 50).

SIRTs mediate lysine deacetylation, which is linked to NAD⁺ hydrolysis (24). Growing evidence suggests that SIRTs are involved in the control of metabolism, and SIRT1, a well-studied SIRTs member, plays a critical role in regulating the progression of renal disease, including diabetic nephropathy (20). In the present study, we showed that ANG 1–7 treatment normalizes the reduction of renal SIRT1 expression in db/db mice. Several clinical and animal studies revealed that an abnormality in lipid metabolism contributes to the pathogenesis and progression of kidney disease, including diabetic nephropathy (29, 38). Abnormal lipid metabolites trigger cellular dysfunction, known as lipotoxicity, and loss of SIRT1 is associated with abnormal lipid metabolism via the reduction of PPARα (37). Renal TAG levels were increased in db/db kidneys, which were normalized by ANG 1–7 treatment, accompanied by the normalization of SIRT1 and PPARα levels. SIRT1 controls FOXO1 activity via deacetylation. Consequently, the reduction of SIRT1 levels likely contributes to the enhanced acetylation of FOXO1 in db/db mice. Concordant with the upregulation of SIRT1 expression, acetylation of FOXO1 was reduced in response to ANG 1–7 treatment. SIRT1 is also regarded as a controller of ATGL activity through acetylation of FOXO1. The observed alteration in the SIRT1-FOXO1-ATGL pathway is consistent with the beneficial effect of ANG 1–7 on diabetic nephropathy.

In conclusion, ANG 1–7 treatment ameliorates diabetic nephropathy in db/db mice due to reduced inflammation, oxidative stress, and fibrosis. ANG 1–7 also decreased renal lipotoxicity, which is accompanied by the ATGL upregulation correlating with deacylation of FOXO1 via SIRT1. ANG 1–7 treatment represents a promising therapeutic tool for the treatment of diabetic nephropathy.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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