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Attenuating effect of angiotensin-(1–7) on angiotensin II-mediated NAD(P)H oxidase activation in type 2 diabetic nephropathy of KK-A^y/Ta mice

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1Division of Nephrology, Department of Internal Medicine, Jutendo University Faculty of Medicine, Tokyo, Japan; 2Division of Nephrology, Department of Internal Medicine, and 4Division of Nephrology, Department of Internal Medicine, Kyung Hee University Hospital at Gangdong, Seoul; and 3Division of Nephrology, Department of Internal Medicine, Kyung Hee University, Seoul, Korea

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Moon JY, Tanimoto M, Gohda T, Hagiwara S, Yamazaki T, Ohara I, Murakoshi M, Aoki T, Ishikawa Y, Lee SH, Jeong KH, Lee TW, Ihm CG, Lim SJ, Tomino Y. Attenuating effect of angiotensin-(1–7) on angiotensin II-mediated NAD(P)H oxidase activation in type 2 diabetic nephropathy of KK-A^y/Ta mice. Am J Physiol Renal Physiol 300: F1271–F1282, 2011. First published March 2, 2011; doi:10.1152/ajprenal.00065.2010.—ANG-(1–7) is associated with vasodilation and nitric oxide synthase stimulation. However, the role of ANG-(1–7) in type 2 diabetes mellitus is unknown. In this study, we examined the hypothesis that ANG-(1–7) attenuates ANG II-induced reactive oxygen species stress (ROS)-mediated injury in type 2 diabetic nephropathy of KK-A^y/Ta mice. KK-A^y/Ta mice were divided into four groups: 1) a control group; 2) ANG II infusion group; 3) ANG II+ANG-(1–7) coinfusion group; and 4) ANG II+ANG-(1–7)+D-Ala7-ANG-(1–7) (A779) coinfusion group. In addition, primary mesangial cells were cultured and then stimulated with 25 mM glucose with or without ANG II, ANG-(1–7), and A779. The ANG II+ANG-(1–7) coinfusion group showed a lower urinary albumin/creatinine ratio increase than the ANG II group. ANG-(1–7) attenuated ANG II-mediated NAD(P)H oxidase activation and ROS production in diabetic glomeruli and mesangial cells. ANG II-induced NF-κB and MAPK signaling activation was also attenuated by ANG-(1–7) in the mesangial cells. These findings were related to improved mesangial expansion and to fibronectin and transforming growth factor-β1 production in response to ANG II and suggest that ANG-(1–7) may attenuate ANG II-stimulated ROS-mediated injury in type 2 diabetic nephropathy. The ACE2-ANG-(1–7)-Mas receptor axis should be investigated as a novel target for treatment of type 2 diabetic nephropathy.

The recent discovery of the renal renin-angiotensin system (RAS) (41), angiotensin-converting enzyme-related carboxypeptidase (ACE2), and ANG-(1–7) has changed the way the RAS is viewed. ANG-(1–7) is present in kidneys at concentrations comparable to ANG II and is associated with vasodilation, modulation of sodium and water transport, and stimulation of nitric oxide synthase (NOS) (7, 9, 24, 34). The effect of ANG-(1–7) on diabetes is still not clear. In a recent study using the streptozotocin-induced diabetic rat model, ANG-(1–7) infusion prevented diabetes-induced abnormal vascular responses to norepinephrine, endothelin-1, and ANG II in the perfused mesenteric bed and renal arteries. This study also reported decreased urinary albumin excretion in response to ANG-(1–7) (6, 7, 13). Another study in the same streptozotocin-induced diabetic rat model showed that chronic injection of ANG-(1–7) accelerated diabetic renal injury (36). In db/db mice, glomerular expression of ACE2 was increased, and ACE2 inhibitor use showed increased glomerular staining for fibronectin and an extracellular matrix protein (42). However, there have been no studies on the direct effect of ANG-(1–7) on ANG II-induced diabetic glomerular changes.

There is emerging evidence that in diabetic nephropathy, the generation of reactive oxygen species (ROS) is a major factor in the development of diabetes and its associated complications (3, 4). NAD(P)H oxidase is an enzymatic complex that is responsible for ROS production. Several studies have reported that the NAD(P)H oxidase subunits, including p47 phagocyte oxidase (phox), p67 phox, p22 phox, Nox, and Rac, are increased in the diabetic kidney (17, 21). ANG II stimulates upregulation of NAD(P)H oxidase subunits in various types of cells as well as in animal models (20, 38). Recent evidence suggests that ANG II stimulates phosphorylation and activation of MAPK in renal tubular cells, and this stimulation depends on NAD(P)H oxidase-mediated injury (18). ROS-mediated injury is also related to the elevation of the profibrosis cytokine transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), and an increment of mesangial matrix expansion (26). However, there is no study about the possibility of ANG-(1–7) preventing ANG II-induced mesangial matrix accumulation related to ROS in the diabetic condition.

The KK-A^y/Ta mouse line was established in 1969, and these mice are widely used as an experimental model for type 2 diabetes mellitus (28). KK-A^y/Ta mice spontaneously exhibit type 2 diabetes mellitus signs, including hyperglycemia, glucose intolerance, hyperinsulinemia, obesity, and microalbuminuria. The mice also develop renal lesions that show diffuse hyperplasia of the mesangial area with mesangial cell (MC) proliferation, segmental sclerosis, overexpression of TGF-β1, and accumulation of advanced glycation end products and ROS products (22, 27, 39).
In this study, we examined the hypothesis that ANG-(1–7) attenuates ANG II-stimulated NAD(P)H oxidase-mediated glomerular injury in type 2 diabetic nephropathy using KK-A^y/Ta mice as a type 2 diabetic nephropathy model. In addition, we used primary cultured mouse MCs to determine whether ANG-(1–7) could counteract ANG II-induced ROS-mediated TGF-β1 activation.

METHODS

Animals. Thirty-two diabetic KK-A^y/Ta Jcl mice were purchased from CLEA Japan (Tokyo, Japan) and housed individually in plastic cages. During the study, all mice received a diet of rodent pellets (348 kcal/100 g) containing 5.5% crude fat as well as tap water ad libitum. The 32 KK-A^y/Ta mice were divided into four groups of eight mice: the diabetic control group (group 1); the diabetes with ANG II infusion group (group 2); the diabetes with ANG II+ANG-(1–7) coinfusion group (group 3); and the diabetes with ANG II+ANG-(1–7)+A779 coinfusion group (group 4). Chronic subcutaneous infusion of ANG II (100 ng·kg^{-1}·min^{-1}), ANG-(1–7) (100 ng·kg^{-1}·min^{-1}), and A779 (100 ng·kg^{-1}·min^{-1}) was performed in mice from 12 to 16 wk of age using subcutaneous osmotic minipumps (model 2004, Alzet). The drug doses were determined from previous studies (14, 23, 31) and were separated by PAGE on a 10% gel then transferred to a polyvinylidene difluoride membrane by electroblotting. The proteins were analyzed by overnight incubation with the antibodies to the following: p47 phox, p67 phox, p22 phox, TGF-β1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), NOX4, fibronectin, type IV collagen (2 μg/ml, Abcam, Cambridge, UK), total Erk, phosphorylated Erk, total p38, phosphorylated p38, total SAPK-JNK, phosphorylated SAPK-JNK, and CTGF (1:1,000, Cell Signaling Technology). After washing,
the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) diluted 1:10,000 in blocking solution, developed with a chemiluminescence agent (ECL plus, GE Healthcare), and analyzed using the LAS-3000 image analysis software program (Fujifilm, Tokyo, Japan). GAPDH (1:10,000, Abcam) and Na⁺/K⁺-ATPase α1 (1:500, Santa Cruz Biotechnology) was used as an internal control. The blots shown are representative of four blots.

Isolation of total RNA, RT, and real-time PCR. Total RNA was extracted from kidney tissue using TRizol Reagent (GIBCO Invitrogen). Complementary DNA was synthesized using random hexamers (Quantum RNA kit, Ambion, Austin, TX) and Superscript II reverse transcriptase (Life Technologies, Rockville, MD). Using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), real-time PCR was performed in a final volume of 25 μl containing 3 μl of cDNA, 2.5 pmol of each sense and antisense primer, and 12.5 μl of SYBR Green PCR Master mix (Applied Biosystems). The primers used for fibronectin, TGF-β1, and GAPDH amplification were as follows: fibronectin forward 5'-TGG CGT CCT TCA ACT TCT CCT-3', reverse 5'-TGT TTG ATC TGG ACT GGC-3', TGF-β forward 5'-GCA ACA TGT GGA ACT CTA CCA GA-3', reverse 5'-GAC GTC AAA AGA CAG CCA CTC A-3'; TGF-β reverse 5'-CAT TGT GGA AGG GCT CAT GA-3', reverse 5'-TCT TCT GGG TGG CAG TGA TG-3'. Each sample was run in triplicate in separate tubes to permit quantification of target gene expression normalized to GAPDH expression.

Light microscopy. For light microscopy, the tissue was embedded in paraffin, cut into 3-μm sections, and stained with periodic acid-Schiff (PAS) reagent. PAS-stained areas in at least 30 glomeruli from each section were quantified using the KS-400 version 4.0 image analysis system (KS-400, Carl Zeiss Vision, Munich, Germany).

Immunohistochemistry. For immunohistochemistry, each kidney was perfused with PBS, 20% sucrose, and 4% paraformaldehyde at a pressure of ~150 mmHg for 3 min. Cryostat-cut 3-μm kidney sections were air-dried for 10 min and then blocked by incubation with blocking solution (PBS containing 2% bovine serum albumin, 2% fetal bovine serum, and 0.2% fish gelatin) for 60 min. Endogenous peroxidase activity was inhibited by incubation with methanol containing 0.3% H₂O₂ for 10 min. For p47 phox, NOX4, nitrotyrosine, and inducible NOS (iNOs) staining, the primary antibody was diluted 1:50 in blocking solution and incubation was performed at 4°C overnight. The sections were incubated with horseradish peroxidase-labeled antibodies (Dako, Carpenteria, CA); then 3,3-diaminobenzidine (DAB) was added for 2 min, and the slides were counterstained with hematoxylin. Significant DAB staining areas in at least 30 glomeruli from each section were quantified using the KS-400 version 4.0 image analysis system (KS-400, Carl Zeiss Vision).

p47 phox and p67 phox membrane translocation. Membrane translocation of p47 phox and p67 phox from the cytosol was analyzed by immunofluorescence. Cultured MCs were seeded on coverslips and stimulated by ANG II with or without preincubation with ANG-(1–7) and A779 for 15 min. Cells were fixed with 4% paraformaldehyde 12 h after ANG II stimulation. After washing and blocking, the cells were incubated with a rabbit anti-p47 phox antibody at 4°C overnight. After washing, the slides were incubated with FITC-conjugated goat anti-rabbit IgG at 37°C. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed by confocal microscopy (Olympus, Tokyo, Japan).

Measurement of NAD(P)H oxidase activity. We determined NADPH levels as an index of the redox status using a commercial colorimetric system (Biovision). Cultured MCs were stimulated by ANG II with or without preincubation with ANG-(1–7), A779, and apocynin for 15 min. After 12 h, NADPH levels were measured in cell lysates. For the latter, NADP was decomposed by heating at 60°C for 30 min. The corresponding 450-nm optical density measurements were read in a NADPH standard curve to determine concentrations. All the above assays were done in triplicate dishes in at least three independent experiments.

Intracellular ROS detection. 2',7'-Dichlorofluorescin diacetate (DCF-DA) was used to detect intracellular ROS production in cells. The fluorescence of this cell-permeable agent significantly increases after oxidation. DCF-DA was purchased from Sigma dissolved in DMSO. Cultured MCs were stimulated by ANG II with or without preincubation with ANG-(1–7) and A779 for 15 min. After 6, 12, and 24 h, cells were fixed with 4% paraformaldehyde 12 h after ANG II stimulation. After washing and blocking, the cells were incubated with a rabbit anti-p47 phox antibody at 4°C overnight. After washing, the slides were incubated with FITC-conjugated goat anti-rabbit IgG at 37°C. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed by confocal microscopy (Olympus, Tokyo, Japan).

Light microscopy. For light microscopy, the tissue was embedded in paraffin, cut into 3-μm sections, and stained with periodic acid-Schiff (PAS) reagent. PAS-stained areas in at least 30 glomeruli from each section were quantified using the KS-400 version 4.0 image analysis system (KS-400, Carl Zeiss Vision, Munich, Germany).
24 h, with the addition of 2 μM DCF-DA solution for 30 min at 37°C, the fluorescence of DCF-DA was detected using flow cytometry (BD FACSCaliber Flow Cytometry). For image analysis for generation of intracellular ROS, the cells were seeded on a coverslip-loaded six-well plate at 2 × 10^5 cells/well. MCs were stimulated under the same condition as for flow cytometry (30 μM DCF-DA, 30 min at 37°C). After washing with PBS, the stained cells were mounted onto microscope slide in mounting medium (Dako). The images were collected using confocal microscopy (Carl Zeiss).

Measurement of NF-κB activation. Cultured MCs were stimulated by ANG II with or without preincubation with ANG-(1–7) and A779 for 15 min. After 12 h, nuclear extracts were obtained by using a Nuclear Extract Kit (Active Motif). Briefly, cells were collected in the PBS/phosphatase inhibitor solution and lysed in lysis buffer containing 10 mM DTT and a cocktail of protease inhibitors. Solubilized proteins were then separated from cell debris by centrifugation (20 min at 14,000 g). The concentration of nuclear fraction was measured by a Bradford assay, and the protein content was adjusted to have the same concentration in all the samples. NF-κB activation was measured by an NF-κB ELISA kit according to the manufacturer’s recommendations. The levels of NF-κB activation were measured in triplicate by a spectrophotometer (Bio-Rad Smart Spec 3000) at OD450. All the above assays were done in at least three independent experiments.

TGF-β1 assay. The secretion of TGF-β1 from the cultured MCs was quantified using a commercially available ELISA kit (R&D Systems) following the manufacturer’s recommendations. For each experiment, a TGF-β1 standard curve was constructed using varying concentrations of recombinant human TGF-β1 protein. All data were corrected for cell protein content as measured by a BCA assay.

Statistical analysis. All values are expressed as means ± SE. Results were analyzed using a Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences in the Kruskal-Wallis test were confirmed by a Wilcoxon rank sum and Mann-Whitney test (used to compare mean differences); a P value <0.05 was considered significant.

RESULTS

Animal data. In a preliminary study, we infused ANG-(1–7) alone into KK-A^+/Ta mice, but there was no significant change in the urinary ACR compared with the control groups (control group urinary ACR: 559 ± 15 vs. ANG-(1–7) group: 552 ± 50 mg/g), and it appeared to involve individual variations in ANG II levels in the urine and kidneys. Our subsequent experiments thus focused on the impact of ANG-(1–7) on ANG II effects in a diabetic nephropathy animal model. The characteristics of the mice used in this study are shown in Table 1. All animals gained weight during the experimental period of 4 wk, but weight gain was greater in the diabetic control group (group 1). Systolic blood pressure was higher in groups 2, 3, and 4 than in group 1, but there was no significant difference between the ANG II infusion and ANG II + ANG-(1–7) coinfusion groups. There were no significant differences in fasting and casual
Fig. 4. Immunohistochemical staining for reactive oxygen species (ROS) expression in glomeruli. Immunostaining was used to evaluate the expression of p47 phox, NOX4, nitrotyrosine, and inducible nitric oxide synthase (iNOs) proteins in glomeruli. ANG-(1–7) treatment attenuated the increase in glomerular nitrotyrosine and iNOs expression by ANG II. Original magnification ×400. The pictures are representative of samples from the 8 mice in each group. Above the graph are representative images of immunohistochemistry.
blood glucose or serum insulin levels between the groups. Urinary ACR was markedly higher in the ANG II infusion group compared with group 1, whereas the ANG II/H11001 ANG-(1–7) coinfusion group (group 3) showed no difference compared with group 1 (group 1: 559 ± 15, group 3: 615 ± 53 vs. group 2: 893 ± 64 mg/g, P < 0.05). The effect of ANG-(1–7) was reversed by the addition of the ANG-(1–7) antagonist A779 (Fig. 1).

**Fig. 5.** Effects of ANG-(1–7) against ANG II on NAD(P)H oxidase in mesangial cells (MCs). A: NAD(P)H oxidase complex in mesangial cells. p47 phox and p67 phox by Western blotting of membrane fraction protein and semiquantitative analysis by Na+/K+-ATPase α-1; NOX4, and NOX2 Western blotting by whole protein in glomeruli. ANG II (10^{-7} M) was used to promote NAD(P)H oxidase complex expression. The effect of ANG-(1–7) on NAD(P)H oxidase levels was comparable to apocynin. Above the graph are representative images of Western blot analysis. Also shown is translocation of p47 phox (B) and p67 phox (C) in MCs. ANG-(1–7) inhibited the translocation of p47 phox and p67 phox upon ANG II stimulation. D: NAD(P)H oxidase activity in MCs. *P < 0.05 compared with high glucose. †P < 0.05 compared with ANG II and high glucose.

**Fig. 6.** Effects of ANG-(1–7) on ANG II-stimulated intracellular ROS production in MCs. A: 2',7'-dichlorofluorescin diacetate (DCF-DA) detection by flow cytometry analysis. Confluent MCs were stimulated with 10^{-7} M ANG II with or without ANG-(1–7) or A779. DCF-DA fluorescence markedly increased after 12-h stimulation by ANG II. The increased fluorescence intensity value markedly decreased by ANG-(1–7) in addition to ANG II. Above the graph are representative images of flow cytometry analysis. The measurements were made in triplicate, and the values are expressed as the means ± SE. *P < 0.05 compared with high glucose. B: DCF-DA detection by confocal microscopy. ANG-(1–7) reduced the green fluorescence intensity (DCF-DA) induced by ANG II.
A

Control  ANG II  ANG-(1-7)  A779  ANG II + ANG-(1-7)  ANG II + ANG-(1-7) + A779

6 hrs

12 hrs

24 hrs

B

Control  ANG II  ANG II + ANG-(1-7)  ANG II + ANG-(1-7) + A779

Relative DCF-DA fluorescence (%)

0  50  100  150  200  250

6 hrs  12 hrs  24 hrs

Control
Angiotensin II (Ang II)
ANG 1-7
A779
ANG II+ANG-(1-7)
ANG II+ANG-(1-7)+A779

*
Effects of ANG-(1–7) on ANG II-induced mesangial expansion in KK-A^T^ mice. We wished to investigate the effect of ANG-(1–7) on ANG II-induced mesangial expansion, which is one of the most striking characteristics of diabetic nephropathy. The glomeruli in KK-A^T^/Ta mice showed accelerated mesangial expansion that was characterized by an increase in PAS-positive mesangial matrix (Fig. 2A). There were markedly more PAS-positive and nuclei-free mesangial areas in the ANG II infusion group than in group 1, whereas the ANG II+ANG-(1–7) coinfusion group (group 3) showed no difference compared with group 1 (group 1: 100 ± 9, group 3: 120 ± 6 vs. group 2: 230 ± 11, relative mesangial area, % of control, P < 0.05). The effect of ANG-(1–7) was reversed by the addition of A779. To relate these results to the progression of diabetic nephropathy, TGF-β1 and fibronectin mRNA expression was determined in whole kidneys. TGF-β1 mRNA expression was increased significantly in groups 2 and 4 compared with group 1 (2.6- and 3.7-fold, P < 0.05, respectively; Fig. 2B). ANG-(1–7) treatment attenuated this increase (P < 0.05), and the use of A779 inhibited the effect of ANG-(1–7). Results were similar for fibronectin mRNA expression (Fig. 2C).

Effects of ANG-(1–7) on ANG II-stimulated NAD(P)H oxidase in glomeruli. Figure 3 shows a representative immunoblot of protein from the lysate of the sieved glomeruli. ANG II infusion increased membrane fraction of p47 phox expression (groups 2, 3, and 4 compared with group 1: 3.4-, 1.9-, and 4.3-fold increases vs. group 1, respectively, P < 0.05). ANG-(1–7) coinfusion attenuated the increase in p47 phox expression by ANG II (P < 0.05). However, the addition of A779 reversed the effect of ANG-(1–7). There were no significant differences in membrane fraction of p67 phox among the groups. Glomerular NOX4 expression also increased in groups 2 and 4 compared with group 1 (2.3- and 2.4-fold, P < 0.05, respectively). ANG-(1–7) treatment nearly reversed the increases in glomerular NOX4 expression by ANG II (P < 0.05). However, the use of A779 abolished the effect of ANG-(1–7). Expression of p22 phox was not markedly different among the groups.

Immunohistochemical staining for glomerular ROS expression in glomeruli. Immunohistochemical staining of p47 phox and NOX4 in glomeruli showed the same trends as the immunoblot data (Fig. 4). Nitrotyrosine and iNOS expression in glomeruli was increased in groups 2 and 4 compared with group 1. ANG1–7 treatment attenuated the increase in glomerular nitrotyrosine and iNOS expression by ANG II. However, the use of A779 abolished the effect of ANG-(1–7).

Effects of ANG-(1–7) on ANG II-stimulated NAD(P)H oxidase in cultured MCs. Preincubation of MCs with 10^{-7} M ANG-(1–7) in addition to ANG II significantly inhibited the ANG II-stimulated phosphorylation of Erk (2), p38, and SAPK-JNK (phosphorylated Erk, HG+ANG II: 3.13 ± 0.30 vs. HG+ANG II+ANG-(1–7): 0.68 ± 0.13; phosphorylated p38, HG+ANG II: 2.10 ± 0.28 vs. HG+ANG II+ANG-(1–7): 0.80 ± 0.12; phosphorylated SAPK/JNK, HG+ANG II: 14.0 vs. HG+ANG II+ANG-(1–7): 7.6 vs. ANG II: 243.7 ± 22.2 pg/well, P < 0.05). ANG-(1–7) significantly decreased ANG II-stimulated activation of NAD(P)H oxidase activity. This effect of ANG-(1–7) was similar with apocynin (ANG II+ANG-(1–7): 130.8 ± 14.0 vs. ANG II+apocynin: 154.9 ± 26.4 pg/well, P > 0.05). All these effects of ANG-(1–7) were neutralized by A779.

Effects of ANG-(1–7) on ANG II-stimulated intracellular ROS production in cultured MCs. The fluorescence dye DCF-DA was used to detect the ROS production activity of ANG-(1–7) in MCs after ANG II stimulation, with the level of ROS detected by flow cytometry.

The graph moved rightward according to an increase in DCF-DA fluorescence. We observed DCF-DA fluorescence markedly increased after 12 h stimulation of ANG II. The increased fluorescence intensity value was markedly decreased by ANG-(1–7) in addition to ANG II (ANG II: 223 ± 18% vs. ANG II+ANG-(1–7): 117 ± 11% of control, under 12-h stimulation, P < 0.05). A confocal microscope analysis showed that ANG-(1–7) reduced the green fluorescence intensity induced by ANG II (Fig. 6B), thus reflecting a reduction in ROS generation.

Effects of ANG-(1–7) on ANG II-stimulated NF-κB activation in cultured MCs. Figure 7 shows the results observed in terms of NF-κB activation. The assay was performed using nuclear extracts. We found that the intensity of NF-κB revealed as twofold increase in ANG II stimulation. ANG-(1–7) reduced the NF-κB intensity induced by ANG II effectively, and this effect was abolished by A779.

Effects of ANG-(1–7) on ANG II-stimulated phosphorylation of MAPK in cultured MCs. Preincubation of MCs with 10^{-7} M ANG-(1–7) in addition to ANG II significantly inhibited the ANG II-stimulated phosphorylation of Erk (2), p38, and SAPK-JNK (phosphorylated Erk, HG+ANG II: 3.13 ± 0.30 vs. HG+ANG II+ANG-(1–7): 0.68 ± 0.13; phosphorylated p38, HG+ANG II: 2.10 ± 0.28 vs. HG+ANG II+ANG-(1–7): 0.80 ± 0.12; phosphorylated SAPK/JNK, HG+ANG II: 14.0 vs. HG+ANG II+ANG-(1–7): 7.6 vs. ANG II: 243.7 ± 22.2 pg/well, P < 0.05). ANG-(1–7) significantly decreased ANG II-stimulated activation of NAD(P)H oxidase activity. This effect of ANG-(1–7) was similar with apocynin (ANG II+ANG-(1–7): 130.8 ± 14.0 vs. ANG II+apocynin: 154.9 ± 26.4 pg/well, P > 0.05). All these effects of ANG-(1–7) were neutralized by A779.

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Fig. 8. Effects of ANG-(1–7) on ANG II-stimulated phosphorylation of MAPK in MCs. A: cells were preincubated with ANG-(1–7) or A779 at the indicated concentration for 15 min before stimulation with ANG II. ANG-(1–7) in addition to ANG II significantly inhibited the ANG II-stimulated phosphorylation of MAPK. Above the graph are representative images of Western blots analysis. *P < 0.05 compared with high glucose. B: effect of ANG-(1–7) on phosphorylation of p38 in nuclear fraction compared with p38 inhibitor (SB203580) by confocal microscopy. ANG-(1–7) had similar effects on the nuclear phosphorylation of p38 compared with SB203580.
2.18 ± 0.18 vs. HG+ANG II+ANG-(1–7); 1.24 ± 0.13-fold of low glucose, P < 0.05; Fig. 8A). Figure 8B shows the effect of ANG-(1–7) on the nuclear phosphorylation of p38 compared with a p38 inhibitor (SB203580) by confocal microscopy. There was no difference between ANG-(1–7) and SB203580 in the nuclear phosphorylation of p38 in MCs.

Effects of ANG-(1–7) on ANG II-stimulated production of profibrosis cytokine and extracellular matrix protein in cultured MCs. MCs cultured with ANG II for 24 h showed a significant increase in TGF-β1 and CTGF protein expression (Fig. 9A). However, preincubation with ANG-(1–7) in addition to ANG II resulted in a reduction of TGF-β1 and CTGF expression. These effects also showed the same tendency in TGF-β1 production (HG: 159.9 ± 25.6 vs. ANG II: 280.9 ± 33.8 pg/μg, P < 0.05; Fig. 9B). The preincubation of ANG-(1–7) in addition to ANG II resulted in a partial reduction of TGF-β1 production (HG+ANG II+ANG-(1–7): 167.7 ± 23.2 pg/μg, P < 0.05). Coincubation with A779 neutralized the effect of ANG-(1–7). ANG II also upregulated fibrosis-related protein, fibronectin, and type I collagen expression. However, preincubation with ANG-(1–7) in addition to ANG II resulted in a reduction of fibronectin and type IV collagen expression in MCs.

Fig. 9. Effects of ANG-(1–7) on ANG II-stimulated production of profibrosis cytokine and extracellular matrix protein in MCs. A: ANG-(1–7) attenuated TGF-β1, connective tissue growth factor (CTGF), fibronectin, and type IV collagen expression against ANG II in MCs. Above the graph are representative images of Western blots analysis. B: production of TGF-β1 in MCs. Each bar indicates the ratio of TGF-β1 protein concentration to cell lysate protein. The measurements were made in triplicate, and the values are expressed as means ± SE. *P < 0.05 compared with high glucose.
Diabetes is known to activate the RAS pathway in the kidney, but the consequences of inhibiting ANG II-mediated ROS injury by ANG-(1–7) have not been studied for type 2 diabetic nephropathy. Using a type 2 diabetes mouse model, we found that mice coinfused with ANG II + ANG-(1–7) had a lower increase in urinary ACR than mice infused with ANG II alone. In this animal model, ANG-(1–7) attenuated ANG II-mediated NAD(P)H oxidase activation and ROS production in diabetic glomeruli and MCs. These findings were related to improved mesangial expansion and to fibronectin and TGF-β1 production in the diabetic kidney and cultured MCs as against ANG II. We also found that ANG II-induced NF-κB and MAPK activation was attenuated by ANG-(1–7) in the MCs.

ANG II-mediated ROS are important second messengers for the transcriptional effects of ANG II, and NAD(P)H oxidase is the central enzyme complex of ANG II-induced ROS (32, 40). In the kidney, all components of classic NAD(P)H oxidase are widely expressed in renal cells, and p47 phox is highly expressed in glomeruli (15, 19). Satoh et al. (35) reported that NAD(P)H oxidase contributes to glomerular ROS production mediated by tetrahydrobiopterin (BH4) availability in a streptozotocin-induced diabetic rat model. Experimental studies have provided strong evidence that ANG II stimulates intracellular formation of O2·− by upregulating the membrane-bound NAD(P)H oxidase and by facilitating formation of its subunits (10, 16, 38). In this study, we infused type 2 diabetic mice with ANG II and confirmed an increase in p47 phox, NOX4, iNOS, and nitrotyrosine expression in glomeruli. We also observed increased NAD(P)H oxidase activity and DCF-DA in MCs after ANG II stimulation.

In a model of hypertension with reduced nitric oxide (NO) synthesis, chronic treatment with ANG-(1–7) lowers blood pressure and improves renal, cardiac, and vascular function, similar to RAS blockade (5). It is well established that the actions of ANG-(1–7) include release of vasodilatory prostaglandins and NO, as well as potentiation of these effects and release or protection of kinins (8, 25). A recent study showed that ANG-(1–7) attenuates renal NAD(P)H oxidase mRNA expression in hypertensive diabetic rats (7). In the present study, the ANG II + ANG-(1–7) group showed only a mild reduction of systolic blood pressure compared with the ANG II group. Despite this, the ANG II + ANG-(1–7) group showed a lower increase in urinary ACR than did the ANG II group. ANG-(1–7) also attenuated ANG II-induced glomerular p47 phox, NOX4, iNOS, and nitrotyrosine expression; this was related to the reduction of mesangial expansion, renal TGF-β, and fibronectin expression. One of the beneficial effects of ANG-(1–7) in diabetic nephropathy may be initiated from the attenuation of ANG II-induced glomerular ROS injury.

The primary cultured MCs also showed NAD(P)H oxidase activation under ANG II stimulation, and it resulted in an H2O2 increment. Increased ROS is known to modify the activity of tyrosine kinases and phosphatases, activate MAPK, and stimulate transcriptional factors (11, 32). Recent studies in cultured cells or in animal models of ANG II-induced tissue injury further support a critical role of NF-κB in mediating the detrimental intracellular signaling effects of ANG II. Ozawa et al. (29, 30) chronically infused ANG II in rats that had developed renal tubulointerstitial injury or fibrosis. The ANG II significantly increased NF-κB binding activity, as assessed by expression of MCP-1 and TGF-β1 mRNA levels, renal cortical tubulointerstitial macrophage infiltration and collagen deposit, and proteinuria. Because the NF-κB inhibitor parthenolide blocked these effects of ANG II, these results clearly implicate a pivotal role of NF-κB in the development of ANG II-induced renal injury. In our study, we demonstrated that ANG-(1–7) attenuated ANG II-stimulated NF-κB activation. It is thought that there is an important intracellular signaling effect of ANG-(1–7) to ANG II.

A recent study showed that ANG-(1–7) inhibits ANG II-stimulated phosphorylation of MAPK in proximal tubular cells and human endothelial cells, and that these effects were reversed by 9-α-Ala2-ANG-(1–7) (33, 37). ANG II-induced ROS-stimulated phosphorylation and activation of Erk in renal tubular cells depends on NAD(P)H oxidase-mediated O2·− formation (18), and ANG II-induced p38 MAPK activation in vascular smooth muscle cells depends on H2O2 (2). In our study, ANG-(1–7) attenuated the ANG II-induced phosphorylation of MAPK. In this regard, ANG-(1–7) seems to attenuate ANG II-induced phosphorylation of MAPK via various pathways, including ROS modulation, with the result is a reduction of the profibrosis marker TGF-β1 and CTGF production. Zimpelmann et al. (43) recently reported that ANG-(1–7) activates phosphorylation of MAPK and that stimulation of p38 MAPK in particular leads to production of TGF-β1 in NG-conditioned human MC lines. However, in that study, treatment with ANG-(1–7) did not result in a significant increase in phosphorylated p38 under HG conditions compared with NG conditions. Our study had the same result. We also confirmed that ANG-(1–7) itself did not show a change in ROS production and the MAPK pathway. However, the role of ANG-(1–7) with respect to ANG II requires further study to determine whether ANG-(1–7) modulates MAPK under various conditions.

In summary, our findings suggest that ANG-(1–7) may attenuate ANG II-stimulated NAD(P)H dependent ROS-mediated injury in type 2 diabetic nephropathy. In addition, we found that ANG-(1–7) attenuated ANG II-induced ROS-mediated injury in MCs. The ACE2-ANG-(1–7)-Mas receptor axis should be investigated further as a novel target of type 2 diabetic nephropathy treatment.

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DISCLOSURES

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

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