ACE2- Ang 1-7-MAS Axis in Human Diabetic Nephropathy

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Abstract

The angiotensin-converting enzyme 2 (ACE2)-angiotensin-(1-7) [Ang (1-7)]-MAS receptor (MAS) axis might act as a counter-regulatory system against the angiotensin-converting enzyme (ACE)-angiotensin II (Ang II)-AT1 receptor (AT1) axis. We studied renal ACE/ACE2-Ang 1-7-MAS expression in human diabetic nephropathy. The subjects were 17 diabetic nephropathy (DN) patients, 17 healthy kidney donors, and 11 minimal change nephrotic syndrome (MCNS) patients as disease controls. Double immunofluorescent staining of kidney sections for ACE and ACE2, Ang 1-7 and ACE2, or MAS and ACE2 was performed, and the results were observed by confocal microscopy. For MAS, immunostaining, in situ hybridization, and RT-PCR were also performed. The percentage area that was positively immunostained for MAS and the intensity of the staining were evaluated by computerized imaging analysis. The median serum creatinine values of the DN patients, MCNS patients, and controls were 1.1 mg/dl, 0.8 mg/dl, and 0.8 mg/dl, respectively, and the median proteinuria values of the DN and MCNS patients were 3.7 g/day and 6.8 g/day, respectively. ACE2 was mainly detected in the proximal tubules, but was also found in the glomeruli in all subjects. ACE2 co-localized with ACE, Ang 1-7, and MAS in the proximal tubules. Compared with the other specimens, the diabetic patients’ proximal tubules displayed increased ACE and decreased ACE2 expression. Tubular Ang 1-7-expression was downregulated in the DN patients compared with the controls and MCNS patients. Tubular MAS expression (density/pixel) was significantly (P<0.001) downregulated in the DN patients [7.33 (6.26-9.68)] compared with the controls [24.51 (18.06-34.56)] and MCNS patients [23.75 (20.11-25.52)].

Conclusions: The tubular ACE2-Ang 1-7-MAS axis is downregulated in human diabetic nephropathy patients compared with healthy controls and MCNS patients.

Keywords: ACE2; Ang 1-7; MAS receptor; Human; Diabetic nephropathy

Introduction

Angiotensin-converting enzyme 2 (ACE2) was discovered in 2000. ACE2 is a homologue of angiotensin-converting enzyme (ACE) that forms angiotensin 1-7 (Ang 1-7) from Ang II and angiotensin 1-9 (Ang 1-9) from angiotensin I (Ang I) [1,2]. With regard to Ang 1-7, the identification of ACE2 and the demonstration that the MAS receptor (MAS) is involved in its actions have contributed to this heptapeptide being classified as a biologically active member of the RAS cascade [3]. The MAS is a receptor for Ang 1-7 [3]. Within the kidneys, Ang 1-7 is generated by ACE2-mediated degradation of Ang II; sequential cleavages of its precursor Ang I by ACE2 and ACE, or the actions of nephrilysin, thimet oligopeptidase, prolyl oligopeptidase, or other peptidases against Ang I [4]. The recently identified ACE2, Ang 1-7, and the MAS has led to conceptual changes in our understanding of the renin-angiotensin system (RAS). There is a proposal that Ang 1-7 counteracts the vasoconstrictor, growth-promoting, and profibrotic action of Ang II [5]. Actually, ACE2, Ang 1-7, and MAS are expressed at high levels in the kidneys and have been reported to display renoprotective effects in animal studies [4-9].

However, the role of the ACE2-Ang 1-7-MAS axis in human diabetic nephropathy remains uncertain. Thus, we compared the renal ACE/ACE2-Ang 1-7-MAS expression of human diabetic nephropathy (DN) patients, healthy controls, and minimal change nephrotic syndrome (MCNS) patients.

Methods

In this observational case-control study, we investigated 17 patients with type 2 diabetes and overt diabetic nephropathy (DN), 17 healthy kidney transplant donors (healthy controls) who underwent renal biopsy during graft transplantation, and 11 patients with MCNS as disease controls, all of whom were admitted to Toho University Hospital between 2006-2010 and aged 20-65 years old. The inclusion criteria for overt diabetic nephropathy (DN) were based on the presence of type 2 diabetes, macroalbuninuria, and renal pathological findings compatible with diabetic nephropathy. All of our diabetic patients had type 2 diabetes, according to the criteria of The American Society of Diabetes Mellitus, and macroalbuninuria. Macroalbuninuria was defined as a urinary albumin-to-creatinine ratio of more than 300 mg/gCr in 2 specimens. DN was confirmed in all diabetic patients by pathological evaluation of renal biopsy specimens under a light microscope, immunofluorescent staining, and electron microscopy. The exclusion criteria for DN were the presence of renal disease other than DN and taking ACE inhibitors or angiotensin II type I (AT1) receptor blockers within 10 days prior to the renal biopsy. At the time of the renal biopsy, 14 patients were on antihypertensive agents, including 9 on calcium channel blockers; 2 on calcium channel blockers and alpha-blockers; 2 on calcium channel blockers...
and diuretics; and 1 on calcium channel blockers, alpha-blockers and diuretics, while 3 patients were not on any antihypertensive agents. None of the healthy controls or MCNS patients were being treated with antihypertensive agents. As for diabetes treatments, 8 patients were on oral antihyperglycemic agents (3 on gliimepiride, 2 on glimepiride and voglibose, 3 on mitiglinide), 7 were receiving insulin injections, and 2 were being treated with diet therapy alone. Seven out of 8 patients on oral antihyperglycemic agents also received antihypertensive agents. Each subject’s salt intake was standardized to 6 g daily during their hospitalization. Diagnosis of MCNS was made by using established criteria of clinical presentation of nephrotic syndrome at the time of renal biopsy, normal serum complement activity, no clinical or serologic evidence of collagen-vascular disease, relatively benign urine sediment, good response to steroid therapy and appropriate renal biopsy finding of minimal changes under light microscopy, no immunofluorescent immunoglobulin staining, and no marked changes except foot process effacement under electron microscopy. The exclusion criteria for MCNS is presence of taking medications at the time of renal biopsy. The medical records of all subjects from the time of the renal biopsy were reviewed to obtain data regarding age, sex, blood pressure, fasting blood sugar (FBS), hemoglobin A1c (HbA1c), proteinuria, serum albumin, and serum creatinine. The estimated glomerular filtration rate (eGFR) was calculated using the equations for estimated GFR together with a Japanese coefficient [10]. The study protocol was approved by the ethics committee of Toho University School of Medicine, and informed consent was obtained from all subjects.

**Immunofluorescent staining and confocal microscopy**

Double immunofluorescent staining of kidney sections for ACE and ACE2, Ang I-7 and ACE2, or MAS and ACE2 was performed. The following primary antibodies were used for the immunofluorescent immunostaining: anti-human ACE goat antibody (AF929: R&D Systems, Minneapolis, MN, USA), anti-human ACE2 mouse antibody (MAB933: R&D Systems), anti-human Ang 1-7 rabbit antibody (H-002-24: Phoenix Pharmaceutical, Inc., Burlingame, California, USA), anti-human ACE2 goat antibody (sc-54682: Santa Cruz Biotechnology), anti-human ACE2- Ang 1-7-MAS Axis in Human Diabetic Nephropathy. J Nephrol Therapeutic S2. doi:10.4172/2161-0959.S2-005

**Immunohistochemical analysis of MAS expression in kidney specimens**

For MAS, immunohistochemistry was also performed using paraffin-embedded tissues by the indirect enzyme horseradish peroxidase-labeled antibody method with LSAB kit (Dako, Carpinteria, CA, USA). The primary antibody used was anti-human MAS1 goat antibody (sc-54682: Santa Cruz Biotechnology). Kidney sections (4-µm thick) were deparaffinized and rehydrated. Antigen retrieval was performed with a pressure cooker at 98°C in target retrieval solution (Dako, Glostrup, Denmark). The sections were incubated overnight at 4°C with the anti-human MAS goat antibody (sc-54682: Santa Cruz Biotechnology), then washed with PBS and incubated with biotinylated universal (anti-rabbit, anti-mouse, anti-goat) IgG followed by incubation with peroxidase-labeled streptavidin. Peroxidase labeling was identified using a liquid diaminobenzidine substrate-chromogen and sections were counterstained with hematoxylin. Negative control tests were performed by omission of primary antibody and use of non-immune sera.

**Semi quantitative analyses for MAS immunostaining**

The intensity of labeling was measured by the method of Mezzano et al. [12], using a computer-based color image analysis system (Mac Scope v2.5, Mitani, Tokyo, Japan), which incorporates Code Warrior by Metrowerks (Austin, TX, USA). The intensity of staining was calculated from the ratio of suitable binary threshold imaging to the total region of interest (ROI), integrating staining intensity in specific areas. For each sample, a mean value was obtained for tubulointerstitial staining by analyzing all fields in a specimen excluding glomeruli and vessels, and the mean value for glomerular staining was obtained by analyzing all glomeruli in a specimen. The staining score was expressed as density/pixel.

**In situ hybridization analysis of MAS expression in human kidney specimens**

The oligonucleotide probes were derived from a 20-mer sequence of human MAS-1 mRNA. The antisense and sense oligonucleotides for human MAS-1 corresponded to bases 651-670 of the MAS-1 gene [ACC. No. NM_02377]. Non-radioactive in situ hybridization was performed on paraffin-embedded sections from the same biopsy sample using reagents purchased from Pan Path (Amsterdam, Netherlands) as instructed by the manufacturer. Briefly, kidney sections (4 µm in thickness) were deparaffinized and rehydrated. After target retrieval, the slides were hybridized with digoxigenin-labeled oligonucleotide antisense probes (100 µM) at 37°C for 16 h. The probe was detected with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and alkaline phosphatase-conjugated anti-digoxigenin antibodies. The sections were counterstained with nuclear Fast Red. The reaction specificity was confirmed by the disappearance of hybridization signals following incubation with the sense probes.

**PCR for human kidney MAS mRNA**

Total RNA was isolated from kidney biopsy specimens using the RNA easy Mini kit (Qiagen, Hilden, Germany). Samples of 1 µg of total RNA were subjected to oligo-dT primed reverse transcription using Moloney murine leukemia virus reverse-transcriptase (Toyobo, Tokyo, Japan). The expression of MAS mRNA and β-actin mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Specimens were amplified in a DNA Thermal Cycler (Eppendorf, Hamburg, Germany) with Taqman Universal PCR Mastermix and Taqman Gene Expression Assay kits for human MAS1, (Assay ID: 6563084F_xF_10M1).
Hs00267157_SI, Applied Biosystems Foster City, CA, USA) and for human β-actin (Assay ID: Hs00357333_g1, Applied Biosystems), respectively, according to the supplier’s protocol. Thermal cycler conditions contained holds for two minutes at 50°C and ten minutes at 95°C, followed 40 cycles of 15 seconds at 95°C and one minute at 60°C. An aliquot of PCR product was then resolved by electrophoresis on 8% polyacrylamide gels and stained by ethidium bromide to visualize signal intensity. β-actin served as an internal RNA control to allow comparison of mRNA levels among different specimens but precise quantitative analysis by real time PCR was not performed in this study.

Statistical analysis

The data were not normally distributed, and non-parametric tests were performed in all analyses. Data are expressed as median values and 25-75 % interquartile ranges (IQR). Differences among the three groups were assessed using the Kruskal-Wallis test and the post hoc Scheffe test. Gender based differences were assessed using the chi-square test. A P-value of less than 0.05 was considered to indicate a significant difference.

Results

The patients’ clinical data are shown in Table 1. Compared with the healthy controls, the DN patients displayed significantly higher SBP, DBP, FBS, HbA1c, proteinuria and serum creatinine levels, while their serum albumin and eGFR levels were significantly reduced. Compared with the MCNS patients, the DN patients displayed significantly higher SBP, DBP, FBS, HbA1c, serum creatinine, and serum albumin levels, while their proteinuria levels and eGFR were significantly reduced. There were no significant differences in age or the sex ratio among the three groups. Median and IQR value of serum creatinine was 1.1 [1.0-1.8] mg/dl, 0.8 [0.6-1.0] mg/dl and 0.8 [0.7-0.8] mg/dl in DN, MCNS and controls. Median value of proteinuria was 3.7 [1.9-4.7] g/day and 6.8 [6.0-10.2] g/day in DN and MCNS.

Localization of ACE, ACE2, Ang 1-7, and the MAS receptor using immunofluorescent staining and confocal microscopy

In our study, the double staining of ACE and ACE2, Alexa Fluor 488 and Alexa Fluor 546 were conveniently green (ACE) and red (ACE2), respectively, and overlapping domains of expression were yellow (Figure 1). In the healthy subjects, ACE and ACE2 displayed co-localization (yellow) in the proximal tubules (Figure 1A). ACE and ACE2 also displayed weak staining in the glomeruli (Figure 1A). In contrast with healthy subjects, ACE2 staining was extremely decreased in both tubules and glomeruli in DN patients (Figure 1B). The merge of both ACE and ACE2 images showed green color but not yellow in the proximal tubules (Figure 1B, lower right). The MCNS patients displayed similar ACE and ACE2 staining patterns to the healthy controls (Figure 1C).

In the double staining of Ang 1-7 and ACE2, Alexa Fluor 680 and Alexa Fluor 546 were conveniently purple (Ang 1-7) and red (ACE2), respectively, and overlapping domains of expression were pink (Figure 2A). Ang 1-7 was detected in the proximal tubules without marked staining in the glomeruli in all three groups (Figure 2A,B,C). As shown in merged image’s pink color, Ang 1-7 and ACE2 co-localized in the proximal tubules in the healthy controls and MCNS patients (Figure 2A,C), but proximal tubules in merged images did not show pink color in tubules of DN patients (Figure 2B). Tubular Ang 1-7 expression was downregulated in the diabetic kidneys compared with those of the healthy controls and MCNS patients (Figure 2B).

In the double staining of MAS and ACE2, Alexa Fluor 488 and Alexa Fluor 546 was conveniently green (MAS) and red (ACE2), respectively, and overlapping domains of expression were yellow (Figure 3). MAS were detected in the proximal tubules and displayed weak staining in the glomeruli in all three groups (Figure 3A,B, and C). As shown in merged image’s yellow color, MAS and ACE2 also co-localized in the proximal tubules in the healthy controls and MCNS patients. (Figure 3C).
3A and C). The merge of MAS and ACE2 images did not show yellow color in the proximal tubules of DN patients (Figure 3B, lower right). Compared with the healthy controls, the MCNS patients displayed a similar tubular MAS expression level (Figure 3C), whereas the DN patients demonstrated downregulated tubular and glomerular MAS expression (Figure 3B).

Immunohistochemical analysis of MAS receptor expression

Images of immunohistochemical staining of the MAS receptor in representative kidney sections from a healthy control, a patient with diabetic nephropathy, and an MCNS patient are shown in Figure 4. Compared with the kidney specimen from the healthy control, the MCNS patient displayed a similar level of MAS receptor staining, whereas the DN patient’s proximal tubules and glomeruli only displayed weak staining (Figure 4).

Table 2 details the results of our semiquantitative analysis of immunohistochemical renal MAS protein expression. Tubular MAS expression (density/pixel) was significantly (P<0.001) downregulated in the DN patients [7.33 (6.26-9.68)] compared with the healthy controls [24.51 (18.06-34.56)] and MCNS patients [23.75 (20.11-25.52)]. Glomerular MAS expression (density/pixel) was also downregulated in the DN patients 0.84 (0.13-1.69) compared with healthy controls [3.26 (2.36-6.65), p<0.05] and MCNS patients [4.02 (2.47-6.86)], although the difference between DN patients and MCNS patients was not significant. No significant difference in tubular or glomerular MAS staining was detected between the MCNS patients and healthy controls.

Human kidney MAS mRNA expression

MAS mRNA was detected in both the proximal tubules and glomeruli in all subjects by in situ hybridization, but tubular MAS mRNA expression was markedly decreased in the kidneys of the DN patients compared with the kidneys of the healthy controls and MCNS patients (Figure 5). Negative control tests using a sense probe did not show yellow signals (B) reflecting weak ACE2 and MAS staining.

**Table 2:** Semiquantitative immunohistochemical analysis of MAS receptor expression.

| Protein staining | Healthy controls | MCNS | Diabetic nephropathy |
|------------------|------------------|------|----------------------|
| N                | 17               | 11   | 17                   |
| Protein staining [density/pixel] | | | |
| Tubulointerstitial MAS receptor | 24.51 (18.06-34.56) | 23.75 (20.11-25.52) | 7.33 (6.26-9.68)**† |
| Glomerular MAS receptor | 3.26 (2.36-6.65) | 4.02 (2.47-6.86) | 0.84 (0.13-1.69)* |

Data are shown as median values with interquartile ranges in parentheses. *P<0.05 compared to the healthy controls, **P<0.001 compared to the healthy controls, †P<0.001 compared to the MCNS patients

MCNS: Minimal change nephrotic syndrome

**Table 2:** Semiquantitative immunohistochemical analysis of MAS receptor expression.

**Discussion**

Diabetes is known to activate the RAS in the kidney [13,14]. Previously, we and others have suggested that the combination of increased ACE expression and decreased ACE2 expression augment Ang II production, leading to accelerated glomerular injury in human type 2 diabetic nephropathy [4,11,15,16]. However, no reports on renal ACE and ACE2 in the same specimen by double staining have been reported in human type 2 diabetic nephropathy. Furthermore, none of them studied angiotensin 1-7, MAS in human diabetic nephropathy with ACE2 in the same kidney specimens. Our study was performed to clarify the co-localization of ACE2, Ang 1-7 and MAS in human kidney in the same specimen by double immunofluorescent staining and confocal microscopy to identify the significance of ACE2-Ang 1-7-MAS Axis in human type 2 diabetic nephropathy. In contrast with healthy subjects and MCNS patients, proximal tubules in merged images using confocal microscopy from DN patients did not show yellow color indicating co-localization of ACE and ACE2, MAS and ACE2, nor pink color indicating co-localization of Ang 1-7 and ACE2.
However, this may be derived from extremely decreased ACE2 staining but not from different localization of these proteins and ACE2 in DN patients.

ACE2 plays a significant role in modulating the RAS by regulating a number of angiotensin molecules including Ang I, Ang II, Ang 1-7, and Ang 1-9. In general, it is thought that activation of the ACE2-Ang 1-7-MAS axis counteracts the actions of the ACE-Ang II-AT1 axis by inhibiting MAP kinase- or cyclooxygenase-2 (COX-2)-dependent pathways, or stimulating the nitric oxide (NO)-cGMP-dependent pathway [5,6,17].

However, Shalamanova et al. [18] reported that they could not detect MAS mRNA expression in the human kidney, primary human renal proximal tubular cells, or human proximal tubular epithelial (HK-2) cells by RT-PCR, although they did detect it in rat primary proximal tubular cells. Thus, they suggested that the expression of MAS in the kidney might be species-specific [18]. In cultured human proximal tubular cells and human mesangial cells, MAS protein expression has been detected by immunoblot experiments [4,19], and we have also detected MAS protein by immunofluorescent staining and immunohistochemistry and MAS mRNA by in situ hybridization and RT-PCR in human kidney biopsy specimens. The discrepancy between Shalamanova’s findings and ours might have been caused by the differences in the primers and PCR conditions used, and we are confident that the MAS is present in human kidneys.

In all subjects in our study, immunofluorescent and immunohistochemical staining of MAS revealed that it is predominantly expressed in the proximal tubules and weakly expressed in the glomeruli. With regard to the presence of MAS in the glomeruli, some immunofluorescent staining study did not detect MAS protein in the glomeruli [20], while others detected immune reactive signals in the glomeruli of the rat kidney [21] or human mesangial cells [19]. Our study suggests that MAS is expressed in the glomeruli in addition to proximal tubules.

Our study showed using confocal microscopy and double immunofluorescent staining that both Ang 1-7 and the MAS colocalized with ACE2 in the proximal tubules of healthy human kidneys. Moreover, we detected the glomerular localization of ACE, ACE2, and MAS although these proteins were not always colocalized, and glomerular staining of Ang 1-7 was not evident in our immunofluorescent study. The results on Ang 1-7 should be interpreted with caution, since, Ang 1-7 is a bioactive transmitter. The concentration of bioactive transmitter (Ang 1-7) may be difficult to demonstrate by immunofluorescent staining because the half-life is usually calculated by seconds even if it is localized in glomeruli. Our immunofluorescent study detected decreased ACE2, Ang 1-7, and MAS staining in the proximal tubules of DN patients compared with those of healthy subjects and MCNS patients. Semi quantitative analysis of MAS immunostaining also detected significantly decreased MAS expression in the tubules and glomeruli of the DN patients compared with the healthy controls. Compared to the MCNS patients, the DN patients displayed significantly decreased MAS immunostaining in their tubules. It was also decreased in their glomeruli although the difference was not statistically significant. The kidneys of the healthy controls and MCNS patients displayed similar ACE, ACE2, and MAS staining patterns in our double immunofluorescent staining experiment. In addition, our semi quantitative analysis of MAS immunostaining did not detect a significant difference between the healthy controls and MCNS patients. Our results are consistent with those of previously reported animal studies. For example, a Western blotting study showed that STZ-induced diabetic mice displayed reduced levels of Ang 1-7, the MAS receptor, and ACE2 compared with normal mice [22]. Using RT-PCR, Shao et al. [23] also reported that MAS receptor mRNA expression was significantly decreased in diabetic rats compared to the controls.

The ACE-Ang II-AT1 axis acts to raise blood pressure, induce renal vasoconstriction, decrease renal blood flow (RBF) and the glomerular filtration rate (GFR), stimulate proximal tubule sodium transport, and increase urine concentration [5,17]. Conversely, the ACE2-Ang 1-7-MAS axis acts to reduce blood pressure, dilate intrarenal blood vessels, increase RBF and GFR, inhibit proximal tubule transport, and induce diuresis [5,17,24]. The ACE-Ang II-AT1 axis is widely implicated in the pathogenesis and progression of diabetic nephropathy [4,17]. ACE2 overexpression to increase Ang 1-7 production or stimulation of the MAS by Ang 1-7 in the kidneys might act to counter the pathological role of the ACE-Ang II-AT1 axis [25,26]. However, the role of Ang 1-7 in the progression of diabetic nephropathy is not fully understood and might be cell specific [4]. It is reported that Ang 1-7 protects against endothelial function deterioration and Ang II-stimulated proximal tubular injury [6], although Ang 1-7 also activates growth stimulatory pathways associated with the production of TGF-β1 and extracellular matrix proteins in cultured human mesangial cells [19]. Benter et al. [27] suggested that Ang 1-7 plays a renoprotective role against the development of diabetic nephropathy. Moon et al. [28] also reported that Ang 1-7 had an attenuating effect on angiotensin II-mediated NAD(P)H oxidase activation in type 2 diabetic nephropathy in mice. While, Shao et al. [23] reported that chronic Ang 1-7 injection accelerates STZ-induced diabetic renal injury. Little is known about the roles of Ang 1-7 and MAS in human diabetic nephropathy, but we consider that the downregulation of ACE2 and the MAS might limit the biological effects of Ang 1-7.

The DN patients’ kidney biopsy samples displayed decreased ACE2, Ang 1-7, and MAS expression levels compared with those of the controls but not those of the MCNS patients. Both genetic ablation of ACE2 [29] and pharmacological inhibition [16] of ACE2 in diabetic mice increase albuminuria. However, the fact that the two proteinuric renal diseases examined in this study did not display similar renal ACE2, Ang 1-7, and MAS expression levels might have been due to differences in the pathogeneses and durations of these diseases. Considered together with previous reports on ACE2-Ang 1-7-MAS in proximal tubules [6,27,28], our fining suggest that this decrease is not solely related to proteinuria.
The present study has certain limitations. The number of human renal biopsy specimens that displayed structural distortion of cortical tissue was relatively small. Furthermore, 14 of the 17 DN patients were being treated with antihypertensive agents including Ca channel blockers, alpha-blockers, and/or diuretics, which might have influenced the intrarenal RAS.

Conclusion

ACE/ACE2-Ang 1-7-MAS co-localize in human proximal tubules. The tubular ACE2-Ang 1-7-MAS axis is downregulated in human diabetic nephropathy patients compared with healthy controls and MCNS patients.

Acknowledgment

The authors thank Mr. Naoji Masuya of Toho University School of Medicine for providing expert statistical advice. This study was presented at the Renal Week Conference (American Society of Nephrology) in November 2010. Denver, CO, USA.

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