Original Article
Telmisartan protects 5/6 Nx rats against renal injury by enhancing nNOS-derived NO generation via regulation of PPARγ signaling

Rong Zou1,2*, Yong He1,3*, Yue-Qiang Li1, Ming Han1, Zu-Fu Ma1, Xiao-Cheng Liu1, Rui Zeng1, Jv-Fang Shao1, Yan-Chao Guo1,4, Xiao-Yu He4, Ping Yang4, Gang Xu1, Cong-Yi Wang4, Ying Yao1

1Department of Nephrology, Tongji Hospital, 2Department of Nephrology, Wuhan Integrated TCM and Western Medicine Hospital, 4The Center for Biomedical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; 3Department of Nephrology, The Fifth City Hospital of Wuhan, Wuhan 430050, China. *Equal contributors.

Received May 19, 2014; Accepted August 15, 2014; Epub October 11, 2014; Published October 15, 2014

Abstract: A 5/6 nephrectomized (Nx) rat model was employed to address the impact of telmisartan on CKD related renal injury and the underlying molecular mechanisms. It was noted that telmisartan provided protection for rats against 5/6 Nx induced lethality. Telmisartan treated 5/6 Nx rats manifested improved renal function as characterized by the higher GFR but lower urinary albumin, BUN and Scr as compared with that of control rats. Telmisartan treatment also significantly decreased systolic blood pressure and alleviated glomerulosclerosis and interstitial fibrosis. Mechanistic studies revealed that telmisartan possesses the capability to increase NO generation in the kidney. Further studies demonstrated that telmisartan promotes PPARγ expression, by which it specifically enhances nNOS expression in the kidneys after 5/6 Nx insult. Particularly, blockade of PPARγ signaling by GW9662 abolished the protective effect conferred by telmisartan, indicating that telmisartan induction of renal nNOS expression along with NO generation is dependent on PPARγ signaling. Together, our data support that telmisartan could be a promising drug for treatment of chronic kidney diseases in diverse clinical settings.

Keywords: nNOS, PPARγ, 5/6 nephrectomy, telmisartan, CKD

Introduction

Previous studies have demonstrated that nitric oxide (NO) generation was decreased in humans with chronic kidney disease (CKD) [1], which was further confirmed in animals that intrarenal NO deficiency is accompanied with CKD progression [2, 3]. Therefore, strategies aimed at improving NO deficiency could be an effective approach for treatment or prevention of CKD progression in clinical settings. There is evidence that neuronal nitric oxide synthase (nNOS) is the main resource of NO release in the kidney, and renal NO deficiency in diseased condition is associated with reduced nNOS expression or activity [4, 5]. Therefore, reduced cortical NO synthesis secondary to decreased nNOS expression might contribute either to the increased intraglomerular pressures, or to the loss of afferent arteriolar autoregulatory function in chronic renal failure (CRF) [6]. Indeed, decreased nNOS expression was noted in animal models with 5/6 renal ablation/infarction [2], chronic glomerular nephritis [7], diabetic nephropathy [8], and chronic renal allograft rejection [9]. In contrast, eNOS expression in response to renal injuries has been found highly variable [10]. For example, animals 2 to 3 wk after 5/6 renal ablation/infarction were manifested with a significant reduction for nNOS rather than eNOS expression [11]. Taken all of these data together, renal cortical nNOS-derived NO deficiency may play a primary role in CKD pathoetiology.

Telmisartan, an Angiotensin AT1 receptor antagonist, can activate peroxisome proliferator-activated receptor γ (PPARγ) due to its highly lipophilic nature and its ability binding to the PPARγ ligand-binding domain [12]. Given that telmisar-
Telmisartan has been found with the capability to increase NO generation by enhancing eNOS expression and activity in a PPARγ-dependent manner [13], we thus assumed that telmisartan may possess similar capacity to enhance nNOS-derived NO production in the setting of CKD by regulation of PPARγ signaling, and we employed a 5/6 nephrectomized (5/6 Nx) rat model to address this assumption.

Materials and methods

Reagents

Telmisartan was obtained from Boehringer Ingelheim (Ingelheim, Germany). Troglitazone, a selective ligand for PPARγ, was kindly provided by Dr. Ming Han (Sankyo, Tokyo, Japan). Polyclonal anti-PPARγ (sc-7196), anti-nNOS (sc-136006), anti-iNOS (sc-8310), and anti-eNOS (sc-654) antibodies were purchased from Santa Cruz Biotechnology (California, USA), while horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit immunoglobulin were from Dako (Glostrup, Denmark). The ECL detection system was from Pierce Biotechnology (Rockford, USA). GW9662, a specific PPARγ antagonist, was from Cayman chemical (Michigan, USA).

Animals and treatments

Normal male Sprague-Dawley rats (6 wk-old, 180-200 g) were purchased from the Department of Laboratory Animal Science Center at the Tongji Medical College. The animals were housed and acclimatized in a light-controlled (12 h/12 h light/dark) animal care unit for 10 days before the experiment. All studies were conducted in accordance with NIH guidelines for the use of experimental animals and were approved by the Tongji Medical College Animal Care and Use Committee (ACUC).

For surgical procedures, the animals were anaesthetized with i.p. injection of pentobarbital (40 mg/kg body weight). After exposure of the right kidney by midline laparotomy, the right renal artery, vein, and ureter were ligated with silk, and the entire kidney was next removed. The poles of the left kidney were removed using a Bovie. Sham rats were undergone a similar procedure except that the kidneys were only touched with the instruments.

Upon the development of hypertension and renal insufficiency (around 30 days after surgery), the animals were randomly divided into 4 groups with each containing 8 animals: 1) 5/6 nephrectomized (5/6 Nx) rats without treatment (M group); 2) 5/6 Nx rats treated with telmisartan (5 mg/kg/d) (T group); 3) 5/6 Nx rats treated with telmisartan (5 mg/kg/d) and GW9662 (0.5 mg/kg/d) (W group); and 4) 5/6 Nx rats treated with troglitazone (4 mg/kg/d) (R group, controls). Sham operated rats were used as additional controls (S group). Clinical and laboratory data including bodyweight (BW), tail-measured systolic blood pressure (SBP), glomerular filtration rate (GFR), blood urea nitrogen (BUN), C-reactive protein (CRP) and serum creatinine (Scr) were assayed for all animals. The study was terminated once 37.5% mortality was achieved in the untreated 5/6 Nx rats (3 months after surgery), and by then, the animals were sacrificed to collect kidneys for experimental purpose.

Assessment of renal NO levels

Nitrate and nitrite (NOx) levels in the renal homogenates and plasma were determined to assess NO generation. Detection of NOx was performed using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s protocol.

Histological analysis and immunohistochemistry

Renal sections were prepared and subjected to H&E, PAS and Masson staining as previously described [14, 15]. A semi-quantitative score was used to assess the severity of glomerular lesions as reported [16]. In brief, the degree of glomerulosclerosis (GS) and mesangial expansion (ME) lesions were classified from zero to four according to the percentage of injury in the glomeruli (0=0%, 1=25%, 2=50%, 3=75% and 4=100% injury, respectively). Around 30 glomeruli in each category were assessed by two pathologists in a blinded fashion. Interstitial matrix deposition was assessed after Masson-Trichrome staining using an image analyzing system (Quantimied 600; Leica, Benzheim, Germany) by comparing the green stained interstitial area (connective tissue) over the entire interstitial area in a particular section [17]. Immunostaining of renal sections with antibodies against PPARγ (1:100 dilution) or nNOS
Telmisartan regulates PPARγ/nNOS signaling

Western blotting and quantitative RT-PCR analysis

Renal lysates were prepared using a cell disrupter in lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 7.4). Protein concentrations were determined by the Bradford method using a Bio-Rad kit (Montreal, Quebec, Canada), in which bovine serum albumin (Sigma, Missouri, USA) was employed as the standards. Western blot analysis of PPARγ, nNOS, eNOS and iNOS expression in the renal lysates was conducted as reported previously [19], and GAPDH was employed for normalization.

For quantitative RT-PCR analysis, total RNA was isolated from renal tissues using an RNasy kit (Qiagen, Chatsworth, CA), and cDNA was then synthesized in a 20 µl volume containing 4 µl reaction buffer, 0.5 mmol/L of each dNTP, 0.5 µmol/L Oligo dT, 25 U RNase inhibitor, and 100 U of M-MLV reverse transcriptase (Qiagen, Chatsworth, CA). RT-PCR was carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, USA) with primers specific for PPARγ (5'-CAA GGG TGC CAG TTT CG-3', 5'-CAG CAG GTT GTC TTG GAT G-3', 396bp) and nNOS (5'-CCC AAT GTA ATT TCT GTT CGT-3', 5'-CCA GGG CAC TGT CAT AGC T-3', 208 bp). GAPDH was used as an internal control (5'-GTG CTG ATG TCT CGG CTG AG-3', 5'-GTG TTC TGA GTG GCA GTG AT-3', 301 bp). The resulting PCR products were separated on 2% agarose gels, followed by densitometry analysis using a Chemilumager 5500 system (Alpha Innotech, San Leandro, CA) as reported [20].

Statistical analysis

Data were expressed as mean±SD, and difference between each group was compared by one-way ANOVA. Multiple comparisons were conducted using the Student-Newman-Keuls post hoc test. All statistical analyses were carried out by using the SPSS 13.0 software (SPSS Inc. Chicago, IL, USA). In all cases, p < 0.05 was considered with statistical significance.

Results

Mortalities of experimental rats in each study group

All experimental rats showed good tolerance for telmisartan treatment, and no perceptible side effect relevant to telmisartan toxicity was noted. As expected, all 8 sham-operated rats in the S group survived through the experimental period. It was noted that 3 animals died (37.5%) in the untreated 5/6 Nx rats (M group), and 2 died (25%) in telmisartan combined with GW9662 treated rats (W group) and troglitazone treated rats (R group). In contrast, only 1 rat (12.5%) died in telmisartan treated T group, which is 2-fold lower than that of animals in the M group, suggesting that administration of telmisartan provided protection for rats against 5/6 Nx-induced lethality.

Assessment of renal function

All 5/6 Nx animals after 30 days of surgery manifested a marked decrease of GFR and a significant increase of urinary albumin excre-
Telmisartan regulates PPARγ/nNOS signaling than that of sham-operated animals (S group). However, these 5/6 Nx rats showed similar GFR at the beginning of drug treatment, while by the end of drug treatment (after 12wks of treatment), 5/6 Nx rats treated with either telmisartan (T group) or troglitazone (R group) manifested a significantly higher GFR and lower urinary albumin than that of untreated 5/6 Nx rats (M group). Significantly higher body weight was also noted in 5/6 Nx rats after treatment with telmisartan (T group) or troglitazone (R group). In line with these data, rats from T and R group manifested much lower levels of BUN and Scr as compared with that of rats from untreated M group. However, no significant difference was noted between rats from sham-operated S group and untreated 5/6 Nx rats from M group as well as animals from T, R and W groups in terms of CRP, blood glucose, and plasma Na⁺ (Table 1). Importantly, those data consistently support that addition of GW9662 attenuated the protective effect conferred by telmisartan.

Blood pressure was also assessed via the tail-cuff method during the treatment weeks of 0, 4, 8, and 12 (Figure 1A). As expected, 5/6 Nx rats without treatment (M group) were characterized by the significantly higher systolic blood pressure. Unlike its impact on body weight, GFR, BUN and Scr, troglitazone treatment failed to show a perceptible effect on the control of systolic blood pressure. Importantly, 5/6 Nx rats treated with either telmisartan (T group) or telmisartan combined with GW9662 (W group) were manifested with significantly lower systolic blood pressure than that of rats from M or R group.

**Results for histological analysis**

To further confirm the above results, renal sections from rats of each group were next subjected to histological analysis. Representative HE staining results for rats from each study group are shown in Figure 1B-F. The severity of glomerulosclerosis was then assessed using a semiquantitative scoring system as described earlier. As expected, all 5/6 Nx rats manifested glomerulosclerosis compared with that of rats from sham-operated S group. However, the most severe glomerulosclerosis was noted in
Telmisartan regulates PPARγ/nNOS signaling in rats in untreated M group, while rats from telmisartan treated T group manifested the least severe glomerulosclerosis, and similarly, blockade of PPARγ signaling by GW9662 dampened telmisartan mediated protection (Figure 2A). We further assessed fibrotic area using an image analyzing system. As shown in Figure 2B, treatment of 5/6 Nx rats with telmisartan significantly reduced fibrotic area. Of note, a reduction for glomerulosclerosis (Figure 2A) and fibrotic area (Figure 2B) was also observed in rats from troglitazone treated R group.

**Telmisartan promotes NO generation**

To address the mechanism by which telmisartan protects 5/6 Nx rats against renal injury, we measured renal lysates for the nitrite/nitrate levels. As shown in Figure 2C, treatment of 5/6 Nx rats with telmisartan significantly increased NO levels, indicating that telmisartan promotes NO generation.

**Figure 2.** Assessment of renal function. A. Scores for the severity of glomerulosclerosis. B. Percentage of fibrotic area assessed by an image analyzing system. C. Nitrite/nitrate (NOx) levels in the renal lysates. The data were collected from 3 rats analyzed in each group. S, sham operation rats; M, untreated 5/6 Nx rats; T, 5/6 Nx rats treated with telmisartan; W, 5/6 Nx rats treated with telmisartan and GW9662; R, 5/6 Nx rats treated with troglitazone.

**Figure 3.** The impact of telmisartan treatment on PPARγ expression. A. Quantitative RT-PCR analysis for PPARγ expression. B. Results for immunostaining of PPARγ expression in renal sections. Three rats from each study group were included for the study. Treatment of 5/6 rats with either telmisartan or troglitazone induced significantly higher levels of PPARγ expression in the kidney. S, sham operation rats; M, untreated 5/6 Nx rats; T, 5/6 Nx rats treated with telmisartan; W, 5/6 Nx rats treated with telmisartan and GW9662; R, 5/6 Nx rats treated with troglitazone.
levels to assess NO production in the kidney. Remarkably, rats originated from telmisartan treated T group and troglitazone treated R group showed significantly higher levels of NO generation as compared with that of rats derived from untreated M group (Figure 2C). Similar as above, addition of GW9662, an antagonist for PPARγ, significantly suppressed telmisartan induced NO generation (Figure 2C).

**Telmisartan enhances PPARγ expression**

Given that blockade of PPARγ signaling by GW9662 abolished telmisartan induced NO generation, we next examined the direct impact of telmisartan on PPARγ expression by quantitative RT-PCR analysis. As shown in Figure 3A, 5/6 Nx rats from untreated M group manifested significantly lower levels of PPARγ mRNA than that of rats from sham-operated S group. More importantly, a significant increase for PPARγ mRNA was detected in rats originated from telmisartan treated T group and troglitazone treated R group, while rats treated with telmisartan and GW9662 (W group) manifested similar levels of PPARγ mRNA as that of rats from M group. Together, these results suggest that telmisartan possesses the capability to promote PPARγ expression in the setting of rats with 5/6 nephrectomy.

The above RT-PCR results promoted us to examine PPARγ protein levels after telmisartan treatment, in which we did immunostaining of renal sections from each group of rats with a specific antibody against PPARγ as described.
The intensity of PPARγ immunoreactivity in the glomeruli and renal tubules in representative rats from each group is shown in Figure 3B. In rats from sham-operated S group, PPARγ immunoreactivity was mainly observed in the glomeruli and proximal convoluted tubules in the cortex. The intensity of PPARγ staining in the glomeruli and renal tubules decreased significantly in 5/6 Nx rats from untreated M group, while PPARγ expression was restored in 5/6 Nx rats treated either by telmisartan (T group) or by troglitazone (R group), but addition of GW9662 almost completely abolished telmisartan induced PPARγ expression (W group).

Telmisartan promotes nNOS expression via PPARγ signaling

Given that nNOS is downstream of PPARγ signaling, we thus next examined the impact of telmisartan treatment on nNOS expression. Quantitative RT-PCR analysis revealed that treatment of 5/6 Nx rats with either telmisartan or troglitazone induced high levels of nNOS expression (Figure 4A), which was confirmed by Western blot analysis of renal lysates from each group of rats (Figure 4B). We further conducted immunostaining of renal sections derived from each group of rats. High intensity of nNOS staining was detected in the glomeruli and renal tubular epithelial cells of sections originated from rats with sham surgery (S group), while a significant reduction for the intensity of nNOS staining was noted in untreated 5/6 Nx rats (M group), and similar as above, treatment of 5/6 Nx rats with telmisartan or troglitazone restored nNOS staining (Figure 5).

It is worthy of note, blockade of PPARγ signaling by addition of GW9662 completely abolished telmisartan induced nNOS expression as confirmed by RT-PCR analysis (Figure 4A), Western blot analysis (Figure 4B) and immunostaining (Figure 5), suggesting that telmisartan induces nNOS expression in a PPARγ dependent manner. To further exclude that telmisartan induction of NO generation in 5/6 Nx rats involves eNOS or iNOS other than nNOS, we did Western blot analysis for eNOS and iNOS expression in renal lysates from each group of rats. We failed to detect a perceptible difference between each group of rats in terms of eNOS expres-
Telmisartan regulates PPARγ/nNOS signaling

Am J Transl Res 2014;6(5):517-527

Figure 6. Western blot analysis for detection of eNOS (A) and iNOS (B) expression in the kidney. The same renal lysates prepared above were used for the analysis, and 3 rats from each study group were included. S, sham operation rats; M, untreated 5/6 Nx rats; T, 5/6 Nx rats treated with telmisartan; W, 5/6 Nx rats treated with telmisartan and GW9662; R, 5/6 Nx rats treated with troglitazone.

Discussion

Telmisartan, a classic angiotensin AT1 receptor blocker, has been demonstrated with capability to attenuate glomerular damage [21], but the underlying molecular mechanisms are yet to be fully addressed. Therefore, in the present report we sought to assess the impact of telmisartan on glomerulosclerosis and interstitial fibrosis, and to address the related molecular mechanisms in the setting of rats with 5/6 nephrectomy, a model relevant to chronic kidney disease (CKD). We first noted that telmisartan provides protection for rats against 5/6 Nx induced lethality. In line with this observation, treatment of 5/6 Nx rats with telmisartan improved renal function as manifested by the significantly higher GFR and lower levels for urinary albumin, BUN and Scr as compared with that of untreated 5/6 Nx rats. Similarly, telmisartan treated rats manifested a marked decrease for systolic blood pressure as compared with that of untreated 5/6 Nx rats. Indeed, telmisartan treatment significantly alleviated glomerulosclerosis and interstitial fibrosis as confirmed by histological analysis. Interestingly, parameters such as CRP, blood glucose and plasma Na⁺ are likely not relevant to CKD progression, given that we failed to detect a significant change between telmisartan treated 5/6 Nx rats and other control rats as well as sham-operated rats.

Given that intrarenal NO deficiency has been consistently demonstrated to be a characteristic factor relevant to chronic kidney disease [1,
Telmisartan regulates PPARγ/nNOS signaling

3, 10], we, therefore next, embarked on renal NO production to address the mechanisms by which telmisartan confers protection for 5/6 Nx rats against CKD progression, in which we measured the renal lysates for nitrite/nitrate (NOx) levels to assess NO generation in the kidney. Remarkably, treatment of 5/6 Nx rats with telmisartan resulted in significantly higher NOx levels, indicating that telmisartan prevent rats from 5/6 Nx induced renal injury by induction of NO generation. Particularly, blockade of PPARγ signaling by GW9662 completely abolished the impact of telmisartan on NO generation. This result promoted us to examine the role of telmisartan in PPARγ signaling. Indeed, telmisartan possesses the capability to directly induce PPARγ expression in the kidney as confirmed by both quantitative RT-PCR analysis and immunostaining of renal sections. Together, these results suggest that telmisartan probably induces renal NO generation by enhancing PPARγ signaling.

NO is synthesized exclusively from its precursor L-arginine under catalysis of nitric oxide synthases (NOS), which includes three isoforms, the neuronal NOS (or NOS I), inducible NOS (or NOS II) and endothelial NOS (or NOS III). Those isoforms are encoded by different genes and only share less than 60% structural homology [22]. In general, eNOS is expressed in endothelial cells of almost all blood vessels except the venous system in the kidney. Immunostaining of renal sections further revealed that eNOS is expressed in endothelial cells of glomeruli and peritubular capillaries in the cortex, and in the endothelial cells of vascular bundles in the medulla, but absent in vascular smooth muscle cells and tubular epithelial cells including proximal tubule cells and macula densa cells [23]. In contrast, macula densa cells are the principal site of nNOS expression in the kidney [24]. nNOS mRNA has also been detected by RT-PCR in microdissected outer medullary collecting duct and in the thin limb of the loop of Henle [25]. Unlike the distribution for eNOS and nNOS, iNOS is likely enriched in tubular epithelium, such as the proximal tubule, cortical and medullary thick ascending limb, distal convoluted tubule, cortical collecting duct and inner medullary collecting duct [26].

Based on the above description, the next important question is to demonstrate the particular isof orm of NOS implicated in telmisartan induced NO generation in the setting of rats with 5/6 nephrectomy. We first checked nNOS expression and demonstrated that 5/6 Nx rats from telmisartan treated T group manifested significantly higher levels of nNOS expression as confirmed by quantitative RT-PCR, Western blot analysis and immunostaining, suggesting that nNOS is probably the primary target after telmisartan treatment. To clarify this issue, we next conducted Western blot analysis for eNOS and iNOS expression by using the same renal lysates. No perceptible change for eNOS expression was detected between telmisartan treated 5/6 Nx rats and control rats as well as sham-operated rats, indicating that alteration in eNOS expression is likely not a causative factor in the setting of 5/6 Nx induced CKD. Similarly, telmisartan treatment failed to induce iNOS expression rather tended to suppress its expression. Together, it is likely that telmisartan enhances NO generation in the setting of 5/6 Nx induced CKD by promoting nNOS expression.

To exclusively demonstrate that telmisartan induces NO generation in the kidney by regulating PPARγ signaling, we employed GW9662, a PPARγ specific antagonist, for negative controls. Given that troglitazone is a ligand for PPARγ, we thus used troglitazone as a PPARγ agonist for the study. Indeed, we demonstrated that blockade of PPARγ signaling by GW9662 almost completely dampened the protection conferred by telmisartan, and on the contrary, similar protective effect was noted in 5/6 Nx rats treated troglitazone, suggesting that telmisartan promotes nNOS expression to increase renal NO generation by regulating PPARγ signaling. Unexpectedly, unlike telmisartan, treatment of 5/6 Nx rats with troglitazone failed to decrease systolic blood pressure although troglitazone demonstrated similar effect as telmisartan on the control of GFR, BUN and Scr. The cause for this discrepancy is currently unknown, which could be due to experimental variations. There is evidence that troglitazone, an analogue of troglitazone, inhibits NO generation by repressing iNOS expression [27, 28]. As a result, the discrepancy could be also associated with troglitazone repression of iNOS expression in our experimental setting, and further studies would be necessary to clarify this conflicting issue.

In summary, we demonstrated feasible evidence that treatment of rats with telmisartan provides protection against 5/6 nephrectomy...
induced renal injury, which involves promotion of nNOS expression along with increased NO generation in the kidney. Moreover, telmisartan induction of nNOS expression is dependent on its impact on PPARγ signaling. Together, these data support that telmisartan could be a promising drug for treatment of chronic kidney diseases in diverse clinical settings.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (811-70686, 30800383 and 81130014), the Ministry of Education (311028), the Start-up Funds for oversea returned students (2007-24), and the Clinical Research Grant from Wuhan Municipal Health Bureau (WX10B03).

Disclosure of conflict of interest

The authors declare no competing financial interests.

Address correspondence to: Dr. Ying Yao, Division of Nephrology, Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. E-mail: yaoyingkk@126.com; Dr. Cong-Yi Wang, The Center for Biomedical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. E-mail: wangcy@tjh.tjmu.edu.cn

References

Telmisartan regulates PPARγ/nNOS signaling


