Original Article

Telmisartan counteracts TGF-β1 induced epithelial–to–mesenchymal transition via PPAR-γ in human proximal tubule epithelial cells

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Abstract: Chronic renal failure (CRF) mainly results from kidney fibrosis. Epithelial-to-mesenchymal transition (EMT) occurs in stressed tubular epithelial cells and contributes to renal fibrosis. Transforming growth factor-β1 (TGF-β1) has been shown to initiate and complete the whole EMT process. Peroxisome proliferators-activated receptor-γ (PPAR-γ) exerts anti-inflammatory, anti-fibrotic and vaculo-protective effects on different renal diseases. Telmisartan is a member of angiotensin II (Ang II) receptor blocker (ARB) family. Recent studies show that Telmisartan has a partial agonistic effect on PPAR-γ. Therefore, we tested the hypothesis that Telmisartan reverses the progression of induced EMT by TGF-β1 in cultured human renal proximal tubular epithelial (HK-2) cells. Cultured HK-2 cells were treated with TGF-β1 (3 ng/ml), a combination of TGF-β1 and Telmisartan (10-200umol/L) and a combination of TGF-β1, Telmisartan and GW9662, a PPAR-γ antagonist for 48 hours. EMT was determined by quantitative real-time PCR analysis of E-cadherin (E-cad), Connective Tissue Growth Factor (CTGF) and PPAR-γ transcript expression and immunocytochemical analysis of E-cad, α-Smooth Muscle Actin (α-SMA) and PPAR-γ protein expression. TGF-β1 induced phenotypic EMT in cultured HK-2 cell line via significantly reduced E-cad expression and significantly increased CTGF, α-SMA expression in association with the loss of epithelial morphology. Telmisartan reversed all EMT markers in a dose-dependent manner which was inhibited by PPAR antagonist GW9662. In the present study, it was suggested that Telmisartan attenuated TGF-β1 induced EMT by agonistic activation of PPAR-γ.

Keywords: PPAR-γ, telmisartan, epithelial-to-mesenchymal transition, proximal tubular epithelial cells, TGF-β1, GW9662

Introduction

Epithelial-mesenchymal transition (EMT) is a process of epithelial cells losing their phenotypic markers and characteristics and acquiring the phenotypic features of mesenchymal cells. The occurrences of EMT are the results of persistent vicious stimuli as injuries and reactions after injuries. EMT is hot topic recently since it involved in many pathologic processes as inflammation, fibrosis tumorigenesis and metastasis.

For instance, EMT occurred in kidney is one of the key mechanisms arousing kidney fibrosis [1-3]. In details, tubular EMT happens in losing epithelial markers such as E-cadherin (E-cad) and acquiring mesenchymal features such as α-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF) [4-7]. Also, tubular epithelial cells lose their epithelial phenotype and become elongated myofibroblast-like phenotype, as well as obtain the capacity of migrating into the peritubular interstitium via the damaged tubular basement membrane (TBM). EMT is thought to be driven by extracellular stimuli, transformation growth factor β1 (TGF-β1) is one of the major player [8-11]. Interestingly, if the surrounding pathogenic factors are removed timely EMT has potential possibility to be reversed [12]. Beyond delaying the inevitable onset of end-stage kidney disease cessation and regression of EMT are ideal schemes of clinical treatment. Realizing the process of EMT is essential in
PPAR-γ and epithelial-to-mesenchymal transition

establishing therapeutic strategies for progressive renal failure, the pathway to counteract and reverse EMT seems to be focused.

Peroxisome proliferators-activated receptors (PPARs) are members of nuclear receptor superfamily with 3 isotypes existed in mammals: PPARα, PPARβ and PPAR-γ [13]. Steroid, thyroid and retinoid hormones are ligands for the receptors. PPAR-γ is highly expressed in adipose tissue; its activation plays a key role in increasing systemic insulin sensitivity. PPAR-γ agonists are clinically used in the treatment of type2 diabetes mellitus and metabolic syndrome. PPAR-γ has been found constitutively expressed in many other tissues like in kidney, for example, of the inner medullary collecting ducts, proximal tubules, thick ascending limb of Henle’s loop, it has been thought to play a protective role for the organ [14]. Also PPAR-γ expression in macrophages and lymphocytes suppresses inflammatory responses and PPAR-γ agonists inhibit the production of proinflammatory cytokines and regulate the process of inflammation by activating this nuclear receptor [15]. More interested, PPAR-γ mediates cellular differentiation, antitumorigenic activity in various tumor types [16, 17] and EMT [18, 19].

Telmisartan is a highly selective angiotensin II type 1 (AT1) receptor blocker (ARB) for treatment of cardiovascular diseases as hypertension. In 2004, it was first identified Telmisartan as partial agonist of PPAR-γ in treatment of hypertension and diabetes [20, 21]. Later on, Telmisartan was reported to connected its functions with PPAR-γ in inflammation [22, 23], cancer treatment [24, 25], as well as used for treating patients with chronic renal diseases [26-28]. Also, it was reported recently special agonist of PPAR-γ activates PPAR-γ in prevention EMT in alveolar epithelial cells [18]. Now, we address the question whether Telmisartan has the capacity of reversing the process of EMT in cultured human kidney proximal tubule epithelial cell HK-2 to counteract EMT-related pathological change as renal fibrosis via PPAR-γ pathway.

Materials and methods

Cell culture and treatment

Immortalized human proximal tubule epithelial cells (HK-2) were obtained from Shenzhen Chinese medicine hospital and maintained in DMEM/F12 medium containing 10% fetal bovine serum (FBS, HyClone, USA) and 1% penicillin/streptomycin solution (HyClone, USA) at 37 °C in 5% CO₂ atmosphere. Cells were used between passages 3 and 20. The HK-2 cells were seeded on standard 6-well culture plates at a density of 150,000 cells/well to approximately 60% to 70% confluence 48 hours prior to treatment. Twenty-four hours prior to treatment, cells were maintained in medium without any additives. TGF-β1(R&D System, USA), TGF-β1+telmisartan (Sigma, USA), TGF-β1+telmisartan+GW9662 (Merck, USA) were then applied to the cells for 48 hours before being harvested for real-time polymerase chain reaction (RT-PCR) or immunofluorescence.

RT-PCR and gene expression analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was generated by reverse transcription 500ng of

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Table 1. Primers used for RT-PCR amplification in the present study*

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>Annealing (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>5'-TCATCAAGACCTGTGCGCCA-3' 5'-GGTTGGGAATCTTTTCCCCCAGT-3'</td>
<td>230bp</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>E-cad</td>
<td>5'-CCCCCATACAGAACTGCAAA-3' 5'-TTCTGGTATCTCCAGGCTAGA-3'</td>
<td>247bp</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>5'-TTGGAATCTTTGAGACGCAAA-3' 5'-GGCCAAACACTGTCAGAGCCTCAG-3'</td>
<td>220bp</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CTCCGGGAAACTGGCGCTG-3' 5'-ACAAAGTGGTCGTTGAGGGCA-3'</td>
<td>351bp</td>
<td>60</td>
<td>30</td>
</tr>
</tbody>
</table>

*CTGF: Connective Tissue Growth Factor; E-cad: E-cadherin; PPAR-γ: Peroxisome Prolifers Activated Receptor γ; GAPDH: glyceraldehyde-3-phosphate dehydrogenase
total RNA in a reaction volume of 10ul random hexamers as a priming agent with PrimeScript RT Master Mix (TAKARA, CHN). One microliters of cDNA was used as template in 20ul PCR reaction. Quantitative real-time PCR was performed using Roche LightCycler 480 Real-Time PCR System with SYBR Premix Ex Taq (TAKARA, CHN). The number of PCR cycles used was determined to be within the linear range of the reactions. Sequences of primers and predicted PCR product sizes were shown as Table 1. Reactions were performed in at least triplicate and analyzed via relative quantitation with data as average fold change compared to no treatment of TGF-β1 after normalization to GAPDH.

**Immunofluorescence labelling of α-SMA, E-cad and PPAR-γ**

HK-2 cells were grown to confluence 60%-70% on sterile cover slips (18×18 mm) under the same conditions described above before the treatment. After 48 hours’ incubation with different drugs, glass slides with cells were washed three times with phosphate-buffered saline (PBS) before fixing in 4% paraformaldehyde/PBS for 30 minutes at room temperature. Next, cells were rinsed in PBS three times and blocked with peroxidase inhibitor (MAIXIN, CHN) for 10 minutes. After blocking, the cells were rinsed in PBS three times and reacted with non-immune serum of intact goat for 10 minutes. Then cells were incubated in diluted primary antibodies separately-mouse anti-human α-SMA (1:50) (R&D Systems) in PBS, mouse anti-human E-cad (1:50) (R&D Systems) in PBS and mouse anti-human PPAR-γ (1:100) (R&D Systems) in PBS overnight at 4 ºC followed by incubation in secondary antibody donkey anti-mouse Alexa Fluor 594 (1:40) (Invitrogen) for α-SMA and PPAR-γ or goat anti-mouse Alexa Fluor 488 (1:40) (Invitrogen) for E-cad at 37 ºC in the dark for one hour. Between each step, cells were washed with PBS adequately. After washing, cells were double-stained with DAPI (1:20) at 37 ºC in the dark for 30 minutes. The slides were mounted using antifade mounting medium and viewed with Leica DM4000 fluorescent microscope (Germany).

**Statistical analysis**

Data are expressed as mean ± standard derivation. Unless stated otherwise, statistical significance was determined using Student’s t-test and statistical significance was achieved when the p value is <0.05.

**Results**

**TGF-β1 increased mRNA level of CTGF and decreased mRNA level of E-cad in HK-2 cells**

HK-2 cells were grown and treated with TGF-β1 (3ng/ml) 48 hours; it had been found that the mRNA level of CTGF increased but the mRNA level of E-cad decreased. The results was consistent the pattern of TGF-β1 induced EMT. The mRNA level of PPAR-γ had not difference between non-treatment and treatment of TGF-β1 (Figure 1).

**Increased mRNA level of CTGF by TGF-β1 was reversed by Telmisartan in HK-2 cells**

In order to examine the function of PPAR-γ in the process, we used PPAR-γ agonist Telmisartan and antagonist GW9662 in the study. HK-2 cells were treated with TGF-β1(3ng/ml) 48 hours in presence of Telmisartan at different concentrations; it was interested that the mRNA level of CTGF decreased when Telmisartan concentration was increasing from 3 to 200µM. To confirm this finding, a PPAR-γ inhibitor, GW9662, was used in treatment. We
To deeply understand the function of PPAR-γ, we were observing the protein expression pattern in stimulation of TGF-β1. As we had known the potential role of PPAR-γ regulating CTGF and E-cad in mRNA level, we questioned how E-cad found that GW9662 (100µM) rebounded the mRNA level of CTGF in presence of Telmisartan (100µM). While incubated with GW9662 (100µM), the effect of increasing E-cad mRNA level by using Telmisartan was blocked (Figure 2).

Also, E-cad mRNA level decreased when HK-2 cells were treated with TGF-β1 (3ng/ml) for 48 hours, but the repression of E-cad mRNA level was inhibited when HK-2 cells were incubated in the presence of Telmisartan (100µM). While incubated with GW9662 (100µM), the effect of increasing E-cad mRNA level by using Telmisartan was blocked (Figure 3).

Based on above finding, we investigated the transcript of PPAR-γ mRNA. As we found when HK-2 cells were stimulated by TGF-β1 (3ng/ml) 48 hours, the mRNA level of PPAR-γ was slightly decreased. After incubated HK-2 cells with TGF-β1 (3ng/ml) in presence of Telmisartan, the mRNA level of PPAR-γ did not change until to 100µM, at the concentration, the mRNA of PPAR-γ backed to the level before TGF-β1 stimulation. At last HK-2 cells were stimulated with TGF-β1 (3ng/ml) in presence of PPAR-γ agonist, Telmisartan (10µM) plus blocker of PPAR-γ (GW9662), the PPAR-γ mRNA level was unchanged (Figure 4).
**Figure 4.** Measurement of mRNA level for PPAR-γ by TGF-β1 plus Telmisartan stimulation in absence or presence of GW9662. Shown quantification of mRNA level of PPAR-γ by RT-PCR measurement after stimulating with TGF-β1 (3ng/ml) plus Telmisartan at different concentrations (0, 10, 30 or 100µM) in absence or presence of GW9662 (0, 10, 30 or 100µM). Data were represented in triplicate experiments ± S.D.

**Figure 5.** Immunofluorescence labelling of HK-2 cells for detecting expression of α-smooth muscle actin, E-cad and PPAR-γ Labeling HK-2 cells with mouse anti-human PPAR-γ (A), mouse anti-human E-cad (B) or mouse anti-human α-SMA (C) followed with donkey anti-mouse Alexa Fluor 594 for α-SMA and PPAR-γ or goat anti-mouse Alexa Fluor 488 for E-cad in non-stimulation, stimulation with TGF-β1 (3ng/ml), TGF-β1 plus Telmisartan (100µM) or TGF-β1 plus Telmisartan (100µM) and GW9662 (100µM). Mouse IgG (mIgG) labeling controls are shown. Bar 20µm.

was expressed in HK-2 cell with TGF-β1 stimulation plus PPAR-γ agonist or antagonist. We found E-cad protein level was regulated by Telmisartan and GW9662 as the same pattern as in its mRNA level, that was, Telmisartan rescued E-cad expression in EMT process and the rescuing were abolished by PPAR-γ inhibitor, GW9662 (Figure 5B). It has been accepted that
the decrease of E-cad expression is correlated with Epithelial-mesenchymal transition or transdifferentiation (EMT). To confirm our hypothesis, the expression of alpha-smooth muscle actin (α-SMA), commonly used as a marker of myofibroblast transdifferentiation, was checked. α-SMA expression level was also influenced by PPAR-γ agonist and antagonist (Figure 5C). Telmisartan blocked the TGF-β1 induced α-SMA expression, but the effect was attenuated by GW9662.

PPAR-γ expression was observed in HK-2 cells, the expression level did not change in presence of its agonist or blocker or both (Figure 5A). It suggested that the action of PPAR-γ might be performed by functional activation of protein rather than by cis-control of its own transcription and translation expression.

Discussion

In the present study we confirmed the efficacy of TGF-β1 inducing EMT in human proximal tubule epithelium HK-2 cell. The EMT was identified by the observation of dramastical down-regulation of E-cad which is expressed in epithelial cells only, and up-regulation α-SMA and CTGF as mensenchymal specific features. Additionally HK-2 cells acquired another myofibroblastic spindle-shaped morphologic phenotype. It is indicated that HK-2 cell lose its ability for attachment and cell adhesion and possessed tendency of transition to myofibroblast cell. The protein expression patterns were attenuated by addition of Telmisartan and reversed by PPAR-γ blocker GW9662, supporting the idea that Telmisartan functions in PPAR-γ dependent manner.

PPAR-γ is a ligand-activated transcription factor belonging to the nuclear Hormone receptor superfamily. Like other nuclear receptors, PPAR-γ has a modular structure consisting of a DNA binding domain, and two ligand binding domains (LBD) [29]. PPAR-γ binding its agonist forms a complex with another nuclear receptor known as retinoid receptor (RXR), conformation change of the heterodimer receptors allows the PPAR-γ/RXR to recognize a specific DNA response elements (PPRE) in the promoter region of target genes thus to promote mRNA transcription.

The ligand dependent control of transcription of PPAR-γ target genes applies for the pharmacological functions in clinical. Conventional PPAR-γ agonist thiazolidinediones (TZD) are widely used in treatment in type 2 diabetes and metabolic syndrome. Since Telmisartan can bind LBD of PPAR-γ with a site that is different to TZD [30], it is not surprising that Telmisartan possesses unique effects not related to that of conventional TZD. Telmisartan was identified as PPARg partial agonist in 2004 [20, 21], since then it has been recognized recently that Telmisartan as PPAR-γ partial agonists has variety of therapeutic effects in insulin resistant type 2 diabetes, hypertension, injury protection in myocardial infarction and brain ischemia [31, 32], anti-inflammation [33], cancer treatment [24]. Through a PPAR-γ dependent manner, Telmisartan also exerts some beneficial effects on the kidney, to decrease proteinurin, inflammation, renoprotection [34-37] although the molecular mechanism is not well understood.

In summary of the novelty of present study, Telmisartan reversed TGF-β1 induced EMT by blocking accumulation of CTGF and α-SMA and decrease of E-cad expression via activating PPAR-γ pathway. The findings might provide supportive evidence for the mechanism of chronic kidney diseases and for the potential usage of Telmisartan in clinical trials in reversal of human renal fibrosis.

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References


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