Role of Fosinopril and Valsartan on Klotho Gene Expression Induced by Angiotensin II in Rat Renal Tubular Epithelial Cells

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Key Words

Klotho · NRK-52E · Fosinopril · Valsartan

Abstract

Background/Aims: Klotho gene, a new anti-aging gene, is mainly expressed in the kidney tubules. Several studies have found the relationship between klotho and emergence and development of renal diseases. This study set out to explore the role of fosinopril (Fos) and valsartan (Val) on klotho expression induced by angiotensin II (Ang II) in rat renal tubular epithelial cells (NRK-52E). **Methods:** NRK-52E cells were divided into five groups according to the treatment of Ang II, Fos and Val. Transforming growth factor- β_1 (TGF- β_1), p38, phospho-p38 (p-p38), p53, and Sp1 protein expression were determined by immunohistochemical and Western blotting analysis. Klotho expression was detected by reverse transcription-polymerase chain reaction and Western blotting analysis. **Results:** Ang II upregulated TGF- β_1 , p-p38 and p53 expression, and inhibited Sp1 and klotho expression in NRK-52E cells. After the intervention of Fos and/or Val, TGF- β_1 , p-p38 and p53 expression were downregulated, Sp1 and klotho expression were upregulated. TGF- β_1 and p53, Sp1 and klotho expression exhibited a positive linear correlation, respectively. **Conclusion:** We conclude that Fos and Val have a protective role in Ang II-induced renal damage, and it may be through mechanism of inhibiting TGF- β_1 , p-p38 and p53 expression, thus upregulating Sp1 and klotho expression.

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Introduction

Klotho gene, a new anti-aging gene, is expressed predominantly in the kidney and choroid plexus, significantly in renal tubular epithelial cells [1]. Its deletion in mice results in phenotypes that resemble human aging, including shortened life span, arteriosclerosis, ectopic calcification, skin atrophy, osteoporosis, pulmonary emphysema and infertility [2]. These features resemble complications of patients with chronic renal failure. Recent studies have shown the association of klotho gene and renal diseases including hypertensive renal damage, acute renal failure, Institute of Cancer Research (ICR)-derived glomerulonephritis, renal cell carcinoma, chronic renal failure and abnormalities in calcium and phosphorus metabolism [3–8], suggesting that the reduction of renal klotho gene expression is associated with the emergence and development of the pathological process of renal diseases. At present, however, little is known about the effects and mechanisms of renal klotho gene expression in renal diseases.

Numerous studies have shown that the renin-angiotensin system (RAS) plays a pivotal role in the progression of renal diseases, and angiotensin II (Ang II) is the main effector [9, 10]. Blockade of the RAS with angiotensin-converting enzyme inhibitors (ACEI) and/or angiotensin type 1 (AT1) receptor blockers is currently the most potential measure to interfere with Ang II-mediated renal damage, and can abrogate progression of renal dis-

eases in part independent of reduction in systemic blood pressure. Therefore, in the present study, we adopted rat renal tubular epithelial cells (NRK-52E) stimulated by Ang II in vitro and used drug intervention including an ACEI, fosinopril (Fos) and/or an AT1 receptor blocker, valsartan (Val), to investigate the possible physiological role of klotho gene in Ang II-induced renal damage.

Methods

Rat Renal Tubular Epithelial Cell Culture

NRK-52E cells were kindly gifted by Dr. Xue-qing Yu (Department of Nephrology, First Affiliated Hospital, Zhong Shan University, Guangzhou, China) and cultured in DMEM supplemented with 10% FBS. Cells, merged to 80% of the dish, were cultured in media agents after pretreatment with non-FBS DMEM for 12 h. Five groups, control, Ang II (10^{-7} mol/l), Ang II (10^{-7} mol/l) + Fos (10^{-5} mol/l), Ang II (10^{-7} mol/l) + Val (10^{-5} mol/l) and Ang II (10^{-7} mol/l) + Fos (10^{-5} mol/l) + Val (10^{-5} mol/l), were cultured for 24 h.

Immunohistochemical Analysis for Transforming Growth Factor- β_1 (TGF- β_1), p38, Phospho-p38 (p-p38), p53 and Sp1

Cells cultured on slides for 24 h in view of immunohistochemical analysis were fixed with 10% formalin. A commercial SABC kit (Wuhan Boster Biological Technology Co. Ltd., China) was used. Briefly, cells were incubated with the following solutions: (1) 3% H_2O_2 for 15 min to quench endogenous peroxidase activity; (2) 10% non-immunized serum to reduce non-specific binding for 30 min at 37°C; (3) primary specific rabbit polyclonal antibody against TGF- β_1 , p38, p-p38, p53 and mouse polyclonal antibody against Sp1 (the ratios of concentration were all 1:150) solution overnight at 4°C; (4) the slices were incubated with biotinylated secondary antibody against rabbit or mouse 1.5 h at 37°C; (5) SABC reagent solution for 30 min at 37°C; (6) incubated with streptavidin peroxidase until the desired stain intensity was seen at 37°C, and (7) dehydrated through an alcohol series and mounted. All the solutions were diluted by PBS and the slices were washed 3 times (each for 3 min) after each step. Brown and yellow colors in cytoplasm indicated positive results for TGF- β_1 , p38 and p-p38 expression, and in the nucleus for p53 and Sp1 expression. Three randomly selected fields of view under the microscope ($\times 400$) were chosen as samples to detect the expression levels of protein; a positive signal was automatically semiquantified using the absorbance (A) value of positive signaling by a pathology image analysis system (PIPS-2000).

Western Blotting Analysis for TGF- β_1 , p38, p-p38, p53, Sp1 and Klotho

The first step was collecting the harvested cells with 10 μ l lysis buffer ($2 \times$ SDS) per 10^6 cells. All the protein samples were boiled for 5 min and each containing 50 μ g protein was loaded onto a 12.5% SDS-PAGE gel, run and electroblotted onto nitrocellulose filters. Blots were blocked in 5% non-fat milk in PBS for 2 h. The membrane was incubated with 1:300 dilution of rabbit monoclonal anti-p53, p38 (Cell Signaling Technologies, Beverly, Mass., USA), polyclonal anti-TGF- β_1 , klotho antibody (Santa Cruz Bio-

technology, Santa Cruz, Calif., USA), or a 1:1,000 dilution of mouse monoclonal anti-Sp1 (Santa Cruz Biotechnology), rabbit monoclonal anti-p-p38 antibody (Cell Signaling Technologies) overnight at 4°C, and then incubated with horseradish peroxidase-conjugated second antibody for 2 h. Immunoblots were developed using an ECL Western blotting detection system (Amersham).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis for Klotho

RNA was isolated from cells using TRIzol reagent (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. Total RNA was then reverse-transcribed into cDNA according to manufacturer's instructions (Fermentas). The resultant cDNA was stored at -20°C until required. The oligonucleotide primers for klotho cDNA and β -actin were synthesized by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd, China. The sequence of the primers used in the PCR were as follows: klotho: sense, 5'-CAA TGG CTT CCC TCC TTT AC-3' and anti-sense, 5'-AGC ACA GGT TTG CGT AGT CT-3'; β -actin: sense, 5'-CGT TGA CAT CCG TAA AGA C-3', and anti-sense, 5'-TGG AAG GTG GAC AGT GAG-3'. The reaction was conducted using the conditions as follows: denature at 94°C for 30 s, anneal at 56°C for 30 s, extend at 72°C for 1 min, 25 cycles; a final extension at 72°C for 10 min. 10 μ l extended products were then electrophoresed on a 1.5% agarose gel, and the bands were visualized using a UVP high-performance ultraviolet transilluminator (UVP GOS7500, USA), the intensity of the klotho DNA bands (512 bp) was expressed as a percentage of the intensity of the β -actin bands (201 bp) with the aid of Bio-Rad Gel Doc 2000 software.

Statistical Analysis

All values are expressed as mean $\pm$ SE. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Q test. Correlation analyses were performed using Pearson's correlation. $p < 0.05$ was considered significantly different.

Results

Immunohistochemical Detection of TGF- β_1 , p38, p-p38, p53 and Sp1 Protein Expression in NRK-52E Cells

Compared with the control group, the protein expression of TGF- β_1 , p-p38 and p53 was significantly increased in the Ang II group in NRK-52E cells; TGF- β_1 , p-p38 and p53 protein expression in the Ang II + Fos group, Ang II + Val group, and Ang II + Fos + Val group were decreased compared to the Ang II group ($p < 0.05$) (fig. 1A, C, D). The Sp1 protein expression in the Ang II group was significantly lower than the control group, but after the intervention of Fos and/or Val, the Sp1 expression reduced by Ang II was significantly increased ($p < 0.05$) (fig. 1E). There was no significant difference of TGF- β_1 , p-p38, p53 and Sp1 protein expression among the Ang II + Fos, Ang

Fig. 1. Immunohistochemical detection of protein expression in different groups of NRK-52E cells (orig. magnif. $\times 400$). **A** TGF- β_1 ; **B** p-38; **C** p-p38; **D** p53; **E** Sp1. a = Control group; b = Ang II group (Ang II 10^{-7} mol/l); c = Ang II + Fos group (Ang II 10^{-7} mol/l + Fos 10^{-5} mol/l); d = Ang II + Val group (Ang II 10^{-7} mol/l + Val 10^{-5} mol/l); e = Ang II + Fos + Val group (Ang II 10^{-7} mol/l + Fos 10^{-5} mol/l + Val 10^{-5} mol/l). Values are mean $\pm$ SE (n = 5). * Significantly different from the control group (p < 0.05). # Significantly different from the Ang II group (p < 0.05).

II + Val, and Ang II + Fos + Val groups (p > 0.05). There was no significant difference in p38 protein expression among different groups of NRK-52E cells (p > 0.05) (fig. 1B).

Western Blotting Detection of TGF- β_1 , p38, p-p38, p53 and Sp1 Protein Expression in NRK-52E Cells

As compared to the control group, the protein expression of TGF- β_1 , p-p38 and p53 in the Ang II group was

significantly higher; Fos and/or Val treatment abrogated the Ang II-induced incremental increases in TGF- β_1 , p-p38 and p53 protein levels (p < 0.05). Ang II induced significantly more abundant protein expression of Sp1 than the control group; the increased abundance of Sp1 expression was abolished by Fos and/or Val treatment (p < 0.05). There was no significant difference of the TGF- β_1 , p-p38, p53 and Sp1 protein expression among the Ang II + Fos, Ang II + Val, and Ang II + Fos + Val

Fig. 2. Western blotting detection of Sp1, p53, p-p38, p38 and TGF- β_1 protein expression in different groups of NRK-52E cells. a = Control group; b = Ang II group (Ang II 10^{-7} mol/l); c = Ang II + Fos group (Ang II 10^{-7} mol/l + Fos 10^{-5} mol/l); d = Ang II + Val group (Ang II 10^{-7} mol/l + Val 10^{-5} mol/l); e = Ang II + Fos +

Val group (Ang II 10^{-7} mol/l + Fos 10^{-5} mol/l + Val 10^{-5} mol/l). Values are mean $\pm$ SE (n = 5). * Significantly different from the control group (p < 0.05). # Significantly different from the Ang II group (p < 0.05).

groups (p > 0.05). There was no significant difference in p38 protein expression among different groups of NRK-52E cells (p > 0.05) (fig. 2).

RT-PCR Analysis and Western Blotting Detection of Klotho mRNA and Protein Expression, Respectively, in NRK-52E Cells

The mRNA and protein expression of klotho in the control group was low, and was significantly decreased in the Ang II group; Fos and/or Val treatment relieved the reduction of klotho mRNA and protein expression by Ang II (p < 0.05), and there was no significant difference between monotherapy and combination therapy (p > 0.05) (fig. 3).

Analysis of the Relationship between TGF- β_1 and p53 Protein Expression, Sp1 and Klotho Protein Expression

Correlation analysis showed that the protein expression of TGF- β_1 and p53 exhibited a positive linear correlation (r = 0.684; p < 0.05); the protein expression of Sp1 and klotho (r = 0.502; p < 0.05) also showed a positive linear correlation.

Discussion

In this study, we showed two major findings. First, Ang II upregulated the expression of TGF- β_1 , p-p38 and p53, then downregulated Sp1 and klotho expression in

NRK-52E cells. Second, Fos and Val had a protective role in Ang II-induced renal damage, maybe through inhibiting TGF- β_1 , p-p38 and p53 expression, thus upregulating Sp1 and klotho expression.

Ang II, the main peptide of RAS, can activate mesangial cells, tubular cells and interstitial fibroblasts, increase the expression and synthesis of extracellular matrix protein and play an important role in the progression of renal disease [11]. At least some of these effects are mediated by upregulation of TGF- β_1 [12, 13], which plays a causal role in the inflammatory changes and collagen deposition in various tissue [14–16]. It has been demonstrated that Ang II can induce upregulation of TGF- β_1 expression, which can activate the mitogen-activated protein kinases (MAPK) signal transduction pathway of p38 (p38MAPK) and increase p53 protein expression, resulting in organ fibrosis [17–19], some of the effects may be through AT1 receptor [20]. An elevation of Ang II-induced renal damage can be diminished by the AT1 receptor blockers [21]. All these findings suggesting that Ang II regulates the renal fibrotic process by TGF- β_1 /MAPK signal transduction pathway and RAS blockers (ACE inhibitors and AT1 receptor blockers) can prevent the activation of the TGF- β_1 /MAPK signal transduction pathway and renal fibrosis via blockade of Ang II action. In our study, TGF- β_1 , p-p38 and p53 expression were upregulated by Ang II in NRK-52E cells, which may be one of the mechanisms involved in Ang II-induced renal damage.

Recent studies have shown that continuous infusion of Ang II downregulated renal klotho gene expression,

Fig. 3. Klotho mRNA and protein expression in different groups of NRK-52E cells detected by RT-PCR and Western blotting, respectively. a = Control group; b = Ang II group (Ang II 10^{-7} mol/l); c = Ang II + Fos group (Ang II 10^{-7} mol/l + Fos 10^{-5} mol/l); d = Ang II + Val group (Ang II 10^{-7} mol/l + Val 10^{-5} mol/l); e = Ang

II + Fos + Val group (Ang II 10^{-7} mol/l + Fos 10^{-5} mol/l + Val 10^{-5} mol/l). Values are mean $\pm$ SE (n = 5). * Significantly different from the control group ($p < 0.05$). # Significantly different from the Ang II group ($p < 0.05$).

which was an AT1 receptor-dependent, but non-pressor-dependent event. Thus, downregulation of the renal klotho gene expression may have an important role in the development of Ang II-induced renal damage [22, 23]. However, the effects and mechanisms of renal klotho expression in Ang II-induced renal damage are still unknown. According to our previous results, we chose a concentration of 10^{-7} mol/l Ang II to intervene with NRK-52E cells to study the effects of klotho gene in Ang II-induced renal damage (data not show).

The promotor in the 5' flanking region of klotho gene has been successfully cloned and sequenced. The upstream transcriptional starting site of klotho gene lacks a typical TATA or CAAT box, instead it has several binding site sequences of transcription factors such as Sp1. The finding is in concordance with the fact that Sp1 is usually located in a promotor region with no TATA box, but the function feature of these sequences is unknown yet [24, 25]. Therefore, we propose that the Sp1-binding site sequence of the upstream promotor region of klotho gene may be the only one important site needed by Sp1 to regulate klotho gene expression. As one of important transcription factors, Sp1 is involved in the transcription regulation of many cytokines and genes, which is closely related to p53 protein. It was reported that p53 protein could inhibit the transcription regulation activity of Sp1 by binding with Sp1 to form complexes [26, 27], or by decreasing the Sp1-binding site sequence via mutation or gene knockout. Thus, Sp1 may be regulated by p53.

In this paper, we found that Ang II could upregulate the protein expression of TGF- β_1 , p-p38 and p53 in NRK-

52E cells, resulting in a significant inhibition of Sp1 and klotho expression. After the intervention of Fos and/or Val, the expression of TGF- β_1 , p-p38 and p53 was down-regulated, while the expression of Sp1 and klotho gene was increased in NRK-52E cells. The protein expression of TGF- β_1 and p53 exhibited a positive linear correlation, indicating that p53 might be activated by TGF- β_1 . In addition, Sp1 and klotho protein expression exhibited a positive linear correlation, indicating that klotho gene expression was likely to be regulated by Sp1.

One of the mechanisms involved in Ang II-induced renal damage may be as follows: Ang II induces upregulation of TGF- β_1 and p-p38 expression, resulting in p53 activation, and then the activated p53 binds with Sp1 in the nucleus to form complexes, which will interfere with the binding of Sp1 and promotor of klotho gene, resulting in the inhibition of klotho expression. Fos and Val may inhibit TGF- β_1 , p-p38 and p53 expression, thus upregulating Sp1 and klotho expression to relieve Ang II-induced renal damage. Ikushima et al. [28] found that the expression of p53/p21 in klotho gene mutant mice was obviously upregulated, while the induction of klotho gene expression could inhibit the activity of p53/p21. Integrated studies showed that there existed a mutual regulation mechanism between klotho gene and p53, which needed further investigation.

We conclude that Fos and Val have a protective role in Ang II-induced renal damage, and it may be through inhibiting TGF- β_1 , p-p38 and p53 expression, thus upregulating Sp1 and klotho gene expression.

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