Effects of fosinopril and losartan on renal Klotho expression and oxidative stress in spontaneously hypertensive rats

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Abstract: **Objective** To explore effects of fosinopril and losartan on renal Klotho expression and oxidative stress in spontaneously hypertensive rats (SHR) and the mechanisms underlying the protection against renal damage. **Methods** Fifteen male SHRs (22 weeks old) were randomly divided into 3 groups ($n=5$ in each group): a SHR group, a fosinopril group [10 mg/(kg·d)], and a losartan group [50 mg/(kg·d)]. Age-matched Wistar-Kyoto (WKY) rats were chosen for a control group. Eight weeks later, tail arterial pressure, 24 hours urinary protein (Upro), urinary N-acetyl-β-D-glucosaminidase (NAGase) were measured. Renal pathological changes were examined under light microscopy by HE staining. The renal mRNA and protein expression of Klotho were determined by RT-PCR, immunohistochemical staining or Western blot. The levels of total antioxidant capacity (TAOC), malondialdehyde (MDA), Cu/Zn superoxide dismutase (Cu/Zn-SOD), Mn superoxide dismutase (Mn-SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were determined. **Results** The typical pathological characteristics of hypertensive renal damage were observed in the kidney of the SHR group. Compared with the SHR group, the systolic pressure, Upro, and urinary NAGase, the content of MDA and renal pathological damage was reduced while the renal Klotho expression and activities of TAOC, Cu/Zn-SOD, CAT, and GSH-Px were increased ($P<0.05$ or $P<0.01$) in the fosinopril or losartan group. There was no significant difference in renal Mn-SOD level among the 4 groups ($P>0.05$). **Conclusion** Fosinopril and losartan can exert protection against hypertensive renal damage through upregulating Klotho expression as well as reducing oxidative stress.

Key words: fosinopril; losartan; Klotho; oxidative stress; hypertensive renal damage

福辛普利、氯沙坦对自发性高血压大鼠 Klotho 表达及氧化应激的影响

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[摘要] **目的**:观察福辛普利(fosinopril)和氯沙坦(losartan)对自发性高血压大鼠(SHR)肾脏Klotho表达及氧化应激的影响,探讨其对高血压肾损伤的保护作用机制。**方法**:15只22周龄雄性SHR,随机分为3组(5只/组):模型组(SHR组),福辛普利组[10 mg/(kg·d)],氯沙坦组[50 mg/(kg·d)];以22周龄雄性Wistar-Kyoto(WKY)大鼠5只为正常对照,共喂养8周。检测尾动脉收缩压、24 h尿蛋白定量(Upro)、尿N-乙酰-8-D-氨基葡萄糖苷酶(NAG酶)等指标。HE染色观察大鼠肾脏病理改变,RT-PCR、免疫组织化学及Western印迹检测Klotho蛋白及mRNA表达;并检测肾脏总抗氧化能力(T-AOC)、丙二醛(MDA)、铜锌超氧化物歧化酶(Cu/Zn-SOD)、锰超氧化物歧化酶(Mn-SOD)、过氧化氢酶(CAT)和谷胱甘肽过氧化物酶(GSH-Px)水平。**结果**:模型组肾脏出现高血压肾损伤的病理特征。与模型组比较,福辛普利或氯沙坦组收缩压、Upro及尿NAG酶均明显降低,肾脏病理损伤减轻($P<0.05$)。肾脏Klotho蛋白及mRNA表达均明显上调($P<0.05$),同时肾脏MDA含量降低,T-AOC,Cu/Zn-SOD,CAT及GSH-Px活性均较模型组增加($P<0.05$或$P<0.01$),Mn-SOD活性无明显改变($P>0.05$)。**结论**:福辛普利、氯沙坦可通过上调抗衰老基因Klotho在肾脏中的表达,并抑制氧化应激,从而对高血压肾损伤起保护作用。

[关键词] 福辛普利; 氯沙坦; Klotho; 氧化应激; 高血压肾损伤

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In recent years, the role of oxidative stress in the occurrence and development of hypertensive renal damage is attracting an increasing attention. Antioxidant can relieve hypertension-induced renal injury[1]. The Klotho gene is a new gene related to aging, which is mainly distributed to the kidney and brain choroids and has a close relationship with oxidative stress[2-3]. Scholars have discussed functions of Klotho gene in the pathogenesis of various renal diseases[4-5], but it is still not clear. This study set spontaneously hypertensive rats (SHR) as objects, intervened by fosinopril (Fos) and losartan (Los), observed changes in renal Klotho gene expression and oxidative stress indexes, in order to discuss the protective mechanisms of angiotensin-coverting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) towards the kidney.

1 MATERIALS AND METHODS

1.1 Main reagents

Fosinopril (10 mg/tablet, given by Bristol-Myers Squibb Inc., USA); losartan (100 mg/tablet, given by Merck& Co, USA); RNA extraction reagent (Trizol, Invitrogen Inc., USA); reverse transcription kit (Fermentas life science, Lithuania); Taq Mix enzyme (Beijing Tianwei Shidai Technology Limited Company); rabbit anti mouse Klotho polyclonal antibody (Biogical Company, US); rabbit anti mouse GAPDH polyclonal antibody and horseradish peroxidase labeled streptavidin anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., USA); total anti-oxidation capacity (T-AOC), malonaldehyde (MDA), copper-zinc superoxide dismutase (Cu/Zn-SOD), manganese superoxide dismutase (Mn-SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) measuring kit (Nanjing Jiancheng Biological Engineering Research Institute).

1.2 Methods

1.2.1 Experimental animals grouping and intervention

Male spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats (22-week) were purchased in Chengdu Shrek Experimental Animals Researching Institute. Fifteen male SHR were divided into 3 groups according to random number table method (each $n = 5$): a model group (SHR group), a fosinopril group [10 mg/(kg·d), intragastric administration], and a losartan group [50 mg/(kg·d), intragastric administration]. Five 22-week male WKY rats were set as a control group. The model group and the control group were given equal amount of distilled water in the morning by intragastric administration. The rats in each group took food and water freely for 8 weeks.

1.2.2 Sample collection and basic data detection

The body mass, caudal artery systolic pressure, 24 h urine protein (Upro), and Urinary-N-acetyl-

8-D-N-acetylglucosamine (NAGase) were detected before the experiment and in the 8th week of experiment (1 d before executing the rats). On the closure of the experiment, 10% chloralic hydras was given and 2 kidneys were removed and weighted. The envelope of the left kidney was removed by longitudinal incision, and the kidney was fixed in 4% paraformaldehyde solution, embedded with paraffin for further use. The right kidney was washed with physiological saline solution and 0.1% DEPC water, stored in liquid nitrogen for RT-PCR, Western blot, and oxidative stress detection.

1.2.3 HE staining

The embedding kidney tissues were sliced and dewaxed. The slices were dyed for 15 min with hematoxylin and washed with running water, dipped in 1% hydrochloric alcohol and washed it with running water, then added 1% eosin for 1 min and washed with running water. The resinene was used to seal the slices after being dehydrated to transparence. The pathological changes of 5 kidney tubule interstitium fields were observed under general microscope (×200) and taken turns with upper left, upper right, lower left, lower right and the middle, then graded by 8 indexes of renal interstitium injury: renal tubular epithelium vacuolar degeneration, kidney tubular ectasia, kidney tubule atrophy, red cell cast, protein cast, interstitial edema, interstitial cell infiltration, and interstitial fibrosis. The mean value of the 8 indexes was considered as the kidney tubular injury index of the sample.

1.2.4 Immunohistochemical staining

Steps were referred to SP kit direction. Positive results were presented with claybank in the cytoplasm and karyon. Ten fields were randomly selected for each section under 400-time microscope. The results were analyzed with pathological image analysis system PIPS-2020 (Chongqing Tianhai).

1.2.5 Detection of Klotho mRNA expression with RT-PCR

Kidney tissues were minced under low temperature (liquid nitrogen), added TRIzol to thoroughly split, and total RNA was extracted according to kit direction. PCR primers were provided by Shanghai Sangon Limited Company. Amplification conditions were pre-degenerated at 94 ℃ for 5 min; degenerated at 94 ℃ for 40 s, extended at 72 ℃ for 45 s, 32 loop; terminated reaction at 72 ℃ 7 min. The 5 μL PCR products were taken and observed under uviol (UV) lamp after 1.5% agarose gel electrophoresis cataphoresis. Quantitative analyze with gel image analysis system was used to compare the average optical density values of targeted genes (A values).

1.2.6 Detection of Klotho protein expression with Western blot

Precooled lysis solution was taken to kidney tissues and total protein was extracted. Protein density was detected with Bradford method. A total of 30-40 μg protein sample was taken and degenerated at 100 ℃ for 5 min, and transferred to PVDF membrane after SDS-PAGE electrophoresis, then sealed for 2 h under room temperature with PBS containing 5% defatted milk powder. First antibody (1:300) and secondary antibody (1:2 000) were added and incubated under room temperature for 2 h, respectively, then developed by enhanced chemiluminescene. Quantitative analyze with gel image analysis system was used to compare the average optical density values of targeted genes (A values).

1.2.7 Detection of oxidative stress indexes

Parts of freeze kidney were taken and maken to 10% tissue homogenate using precooled physiological saline water under ice-bath. The upper clear liquid was taken after centrifugating at 10 000 r/min for 15 min under 4℃. Activity of T-AOC and GSH-Px was measured by colorimetric method, MDA quantity was by thiobarbituric acid (TBA) method, Cu/Zn-SOD and Mn-SOD activity was by xanthine oxidase method, and CAT activity was by UV spectroscopy.

1.3 Statistical analysis

All the data are presented with mean ± standard deviation ($\bar{x} \pm s$) and processed via software SPSS13.0. ANOVA and LSD-t were adopted among multi-group comparisons. $P < 0.05$ was con-

sidered as statistically significant.

2 RESULTS

2.1 Comparison of general data in rats

There was not significantly different in systolic pressure, Upro and urinary NAGase of the SHR group, fosinopril group and losartan group ($P > 0.05$), which in SHR group was higher than those in the WKY group before the experiment (22-week age). After 8 weeks intervention by fosinopril or losartan (30-week age), systolic pressure, Upro and Urinary NAGase decreased ($P < 0.01$) compared those before the experiment and there was not significantly different between the 2 intervene groups ($P > 0.05$). There was not significantly different in the left renal/body weight (LR/BW) of the 4 groups ($P > 0.05$, Tab. 1).

Tab. 1 General data of the 4 groups before and after the therapy ($\bar{x} \pm s$, $n = 5$)

Groups	Age/week	BP/mmHg	Upro/(mg/24 h)	NAGase/(U/L)	LR/BW/‰
WKY group	22	110.75 ±9.59	12.75 ±4.31	15.24 ±5.67	
	30	112.98 ±7.27	12.46 ±9.45	17.11 ±6.97	3.85 ±0.74
SHR group	22	187.58 ±12.36**	85.54 ±30.50**	40.19 ±9.02**	
	30	189.66 ±21.13**	96.89 ±35.75**	53.32 ±10.51**	3.60 ±0.38
Fosinopril group	22	189.50 ±15.31**	82.01 ±32.38**	45.42 ±9.71**	
	30	134.56 ±13.15△△	54.21 ±28.25△△	44.83 ±9.61△△	3.76 ±0.40
Losartan group	22	183.92 ±11.27**	79.23 ±40.22**	43.86 ±8.26**	
	30	129.24 ±9.85△△	57.90 ±30.16△△	46.05 ±8.77△△	3.54 ±0.67

Compared with the WKY group, **$P < 0.01$; compared with the SHR group, △△$P < 0.01$.

2.2 Pathological changes of rats in each group

HE dye showed that there was of no obvious abnormality in rats kidney structures in the WKY group. In the SHR group, thicking of renal arterioles, ischemia and shrinking of some glomerular capillary, some interstitium inflammatory cell infiltration, vacuole and granular degeneration in renal tubular epithelium were seen, and index of renal tubule interstitium injury obviously increased ($P < 0.01$). In the fosinopril group and the losartan group, renal pathological injury was much lower than that in the SHR group, and index of renal tubule interstitium injury decreased ($P < 0.01$, Fig. 1)

2.3 Renal Klotho mRNA and protein expression

Immunohistochemistry staining showed that Klotho protein expressed in renal tubular epithelium cytoplasm and karyon, mainly located in distal convoluted tubule (Fig. 2). RT-PCR and Western blot showed that in the SHR group, Klotho mRNA and protein expression of kidney obviously decreased compared with that in the WKY group. After fosinopril and losartan intervened, Klotho expression obviously increased compared with that in the SHR group ($P < 0.05$ or $P < 0.01$), and there was not significantly different between the 2 intervene groups ($P > 0.05$, Fig. 3-4).

2.4 Changes in renal oxidative stress indexes

In the SHR group, renal MDA quantity increased dramatically, activity of T-AOC, Cu/Zn-SOD, CAT, and GSH-Px decreased compared with those in the WKY group ($P < 0.01$). After fosinopril or losartan intervened, renal MDA quantity decreased, activity of T-AOC, Cu/Zn-SOD, CAT, and GSH-Px increased compared with those in the SHR group ($P < 0.05$ or $P < 0.01$). The increase of GSH-Px in the fosinopril group was more than that in the losartan group ($P < 0.05$). There was not significantly different in Mn-SOD activity in the 4 groups ($P > 0.05$, Tab. 2).

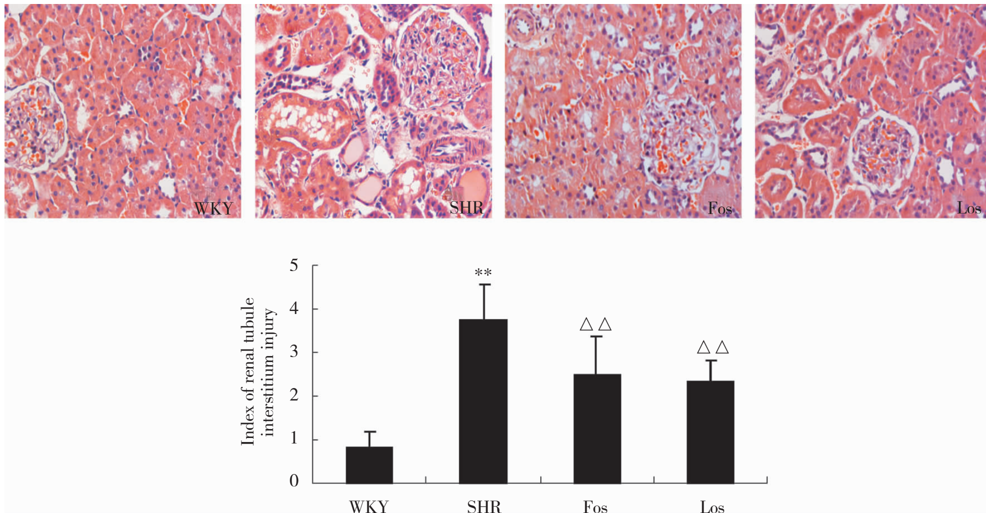

Fig. 1 Renal pathological changes in the 4 groups (HE, ×400). Compared with the WKY group, $**P<0.01$; compared with the SHR group, $\triangle\triangle P<0.01$.

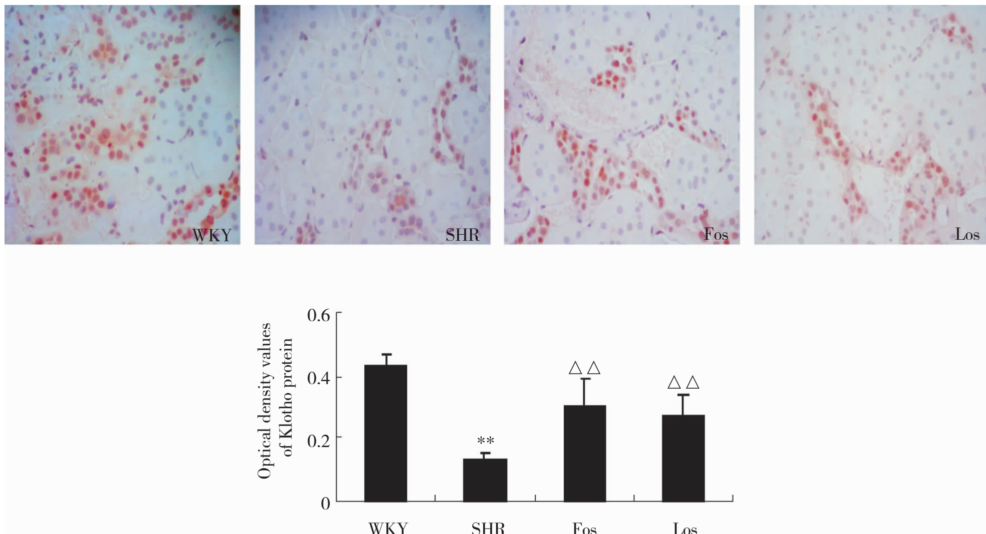

Fig. 2 Renal Klotho protein expression in the 4 groups (Immunohistochemistry, ×400). Compared with the WKY group, $**P<0.01$; compared with the SHR group, $\triangle\triangle P<0.01$.

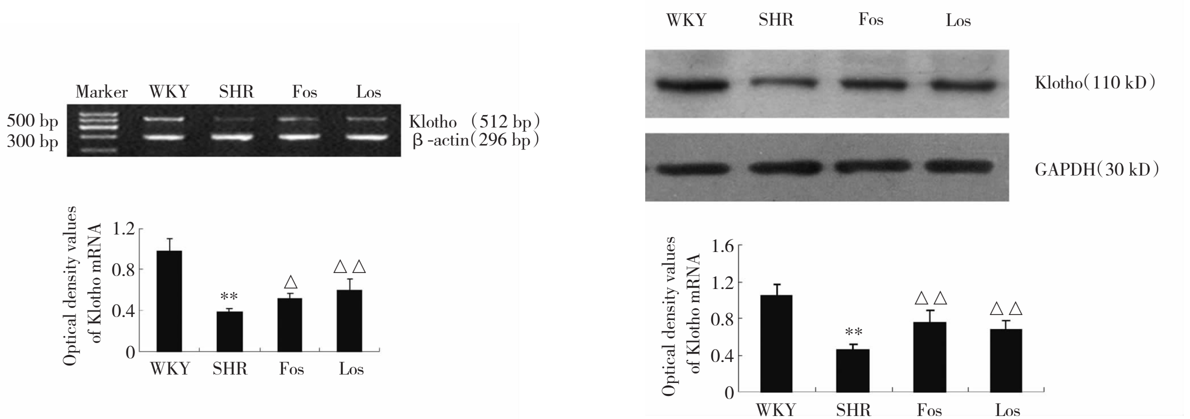

Fig. 3 Renal Klotho mRNA expression in the 4 groups. Compared with the WKY group, $**P<0.01$; compared with the SHR group, $\triangle P<0.05$, $\triangle\triangle P<0.01$.

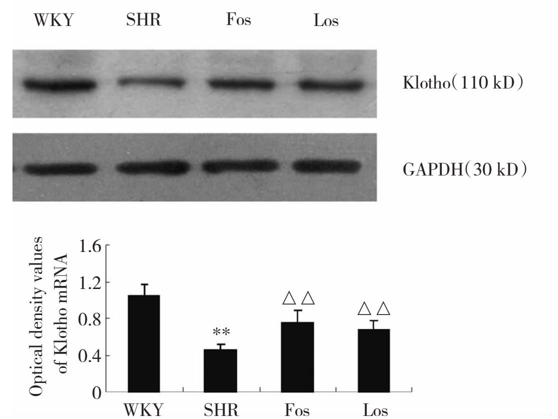

Fig. 4 Renal Klotho protein expression in the 4 groups. Compared with the WKY group, $**P<0.01$; compared with the SHR group, $\triangle\triangle P<0.01$.

Tab. 2 Levels of renal oxidative stress related indexes in the 4 groups ($\bar{x} \pm s, n = 5$)

Groups	T-AOC/(U/mg)	MDA/(nmol/mg)	Cu/Zn-SOD/(U/mg)	Mn-SOD/(U/mg)	CAT/(U/mg)	GSH-Px/(U/mg)
WKY group	1.12 ± 0.14	0.95 ± 0.19	37.05 ± 1.99	16.87 ± 0.49	25.17 ± 2.46	50.64 ± 3.84
SHR group	0.51 ± 0.08**	2.09 ± 0.07**	20.19 ± 0.68**	14.82 ± 1.57	10.46 ± 0.76**	35.77 ± 2.68**
Fosinopril group	0.65 ± 0.03△	1.64 ± 0.13△△	25.84 ± 0.89△△	13.99 ± 1.06	16.93 ± 2.02△△	40.82 ± 2.38△△#
Losartan group	0.80 ± 0.11△△	1.77 ± 0.04△△	27.08 ± 1.75△△	15.23 ± 0.72	15.75 ± 1.33△△	39.10 ± 1.45△

Compared with the WKY group, ** $P < 0.01$; compared with the SHR group, △$P < 0.05$, △△$P < 0.01$; compared with the Los group, #$P < 0.05$.

3 DISCUSSION

Hypertension is a common disease which does great harm to human health, and will finally result in heart function damage, stroke and renal failure. In China, the occurrence of hypertensive renal damage is 20.87%[6]. In the USA, neo-patients who suffered from end stage renal disease caused by hypertension occupies 28% of the total number[7]. In the process of the occurrence and development of hypertensive renal damage, the function of oxidative stress has gained an increasing attention. Oxidative stress is a pathological state caused by the imbalance of the occurrence of reactive oxygen species (ROS) and antioxidation, which plays an important role in the process of hypertensive renal damage and has a good effect in the application of antioxidative stress treatment[8].

Klotho gene is a newly found anti-aging gene in SHR related research. Scholars has discussed the function and status of Klotho gene in the pathogenesis of kidney diseases in the research of SHR, diabetic nephropathy rats, chronic kidney failure and its disorders of calcium and phosphorus metabolism[4-5, 9-10]. Among the numerous molecular mechanisms of Klotho gene, the hottest topic in research is its antioxidative activity. Klotho gene can remove ROS and increase the resistance by activating cAMP signal pathway, suppressing insulin/IGF-1 signal path, and inducing the production of nitric oxide[11-12]. The newest research showed that inducing Klotho expression via Klotho gene adenovirus could delay the process of hypertension and renal damage in spontaneous hypertension rats, whose mechanism may be related to suppressing activity of NADPH oxidase[5]. In accordance with literature reports[13], we found that Klotho gene mainly expressed in renal distal tubular epithelial cells, and obviously decreased in continuous hypertension state, indicating it was involved in hypertensive renal damage.

Based on the importance of oxidative stress in hypertensive renal damage, we examined the levels of oxidative stress related indexes such as T-AOC, MDA, SOD, CAT, and GSH-Px in SHR kidney tissues. T-AOC can reflect antioxidation capacity of the whole body. If many free radicals are not removed, lipids will be peroxide damaged. One of the final products is MDA, which can indirectly reflect damaging degrees of free radicals done on cells. SOD, CAT and GSH-Px are main members of antioxidant system to remove oxygen radicals. The increase of oxidative stress capacity had been confirmed in different hypertension experimental animal models[14-15]. Our research showed renal T-AOC activity of SHR obviously decreased, which confirmed the existence of SHR oxidative stress state. The renal MDA quantity of SHR increased obviously, Klotho expression, antioxidase Cu/Zn-SOD, SAT, CAT and GSH-Px activity decreased compared with the control group, suggesting kidney of SHR is presented with oxidative stress damage, oxidative stress is an important mechanism of hypertensive renal damage, and the decrease of Klotho gene expression may be one of the reasons.

Renin-angiotensin system (RAS) plays an important role in hypertensive renal damage, especially in activition of renal local RAS, which is the key step of the progression of renal disease. Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) can suppress glomerulosclerosis and kidney tubule interstitium damage caused by hypertension through controlling hypertension, improving glomerulus haemodynamics, sup-

pressing oxidative stress, and lessening accumulation of extra cellular matrix[16]. It was found in our experiment that blood pressure, upro and urinary NAGase of SHR obviously increased compared with WKY rats in the same age, and pathological examination showed that SHR was presented with typical characteristics of hypertensive renal damage. Eight weeks after being intervented with fosinopril and losartan to SHR, blood pressure, upro, urinary NAGase obviously decreased and the renal pathological damage was relieved. Also, renal Klotho gene expression, T-AOC activity, MDA quantity, as well as antioxidase Cu/Zn-SOD, CAT, and GSH-Px activity increased. The above mentioned results indicated that fosinopril and losartan can improve anti-aging gene Klotho expression, remove oxygen radicals, and increase anti-oxidation capacity, which may be one of the mechanisms in the treatment of hypertensive renal damage. Although ACEI and ARB are different in the intervention of ways and functional locations, they both can lower blood pressure, reduce urinary protein, up-regulate Klotho gene expression, relieve oxidative stress, which act as a protection for hypertensive renal damage. It is still unknown whether Klotho plays a role in the upstream of oxidative stress reaction when it happens.

In conclusion, the abnormal expression of anti-aging gene klotho and oxidative stress damage existed in kidney of SHR. Besides decreasing blood pressure, fosinopril and losartan can up-regulated Klotho gene expression to suppress oxidative stress reaction, which delays the progression of hypertensive renal damage and protects the function of target organs.

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