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The mechanism of akt-mediated APPL1 in chronic renal fibrosis induced by acute renal ischemia-reperfusion injury

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Abstract

Objective: To observe the protective mechanism of protein kinase B (Akt)-mediated phosphoserine binding domain and leucine zipper motif 1APPL1) on renal fibrosis in mice with renal ischemia reperfusion injury. **Methods**: A total of 24 male C57BL/6 mice were selected to randomly divide into 2 groups: Normal mice-sham operation group (WT-C group) and normal mice-renal ischemia reperfusion group (WT-I/R group). Forty-eight mice with APPL1 gene knockout mice were divided into 4 groups randomly:APPL1 gene knockout/sham surgery group (AK-C group), APPL1 gene knockout/renal ischemia reperfusion group (AK-I/R group), APPL1 gene knockout+Akt inhibitor treatment group (AK-L group), and APPL1 gene knockout+saline treatment group (AK-S group). Except the two control groups of WT-C ad AK-C, the renal ischemia reperfusion injury (IRI) model was prepared in the other groups. After the successful preparation of the model of bilateral renal ischemia reperfusion injury in mice, the AK-L group was intraperitoneally injected with LY294002 mg/kg, once every 24 hours till day 7, and the same dose of normal saline was injected in the AK-S group. At 24h after reperfusion, 6 mice were randomly selected from each group and detected for the concentration of serum BUN and Scr to score renal tubular injury and determine APPL1 expression and Akt phosphorylation levels in the renal tissues. At 14 days after reperfusion, 6 mice in each group were randomly sacrificed to obtain kidney tissues. The degree of renal fibrosis was evaluated; expression of collagen 1, fibronectin, α- smooth muscle actin and APLL1 and Akt phosphorylation levels in renal tissue were determined. **Results:** At 24h after reperfusion, comparisons of serum BUN and Scr concentrations and renal tubular injury scores showed no statistically significant difference between the WT-C group and AK-C group ($P>0.05$). Compared with the WT-C and AK-C groups, the levels of them in the WT-I/R group, the AK-I/R group, and the AK-S group increased ($P<0.05$), and the levels in the AK-L group were the highest ($P<0.05$). At 14 days after reperfusion, the expression of collagen 1, fibronectin and alpha smooth muscle actin in renal tissues and the degree of renal fibrosis showed no significant difference between WT-C group and AK-C group ($P>0.05$). Compared with the WT-C and AK-C groups, the levels of expression of fibrosis-related indices

were increased in the WT-I/R group, the AK-I/R group, and the AK-S group (*P*<0.05), with the highest level in the AK-L group (*P*<0.05). At 24h and 14 d after reperfusion, APPL1 expression and Akt phosphorylation level in renal tissues in the WT-I/R group were up-regulated compared with those in the WT-C group (*P*<0.05). At 24h after reperfusion, compared with the AK-C group, Akt phosphorylation level increased in the AK-I/R group and the AK-S group (*P*<0.05). Compared with the AK-I/R group, Akt phosphorylation level decreased in the AK-L group (*P*<0.05). Conclusions APPL1 mediated by Akt has protective effect on chronic renal fibrosis in mice with renal ischemia reperfusion injury.

Keywords: protein kinase B ; fibrosis ; kidney ; ischemia reperfusion; signal transduction protein

1. Introduction

Some patients with acute renal injury (AKI) can progress to chronic kidney disease (CKD)[1] ischemia-reperfusion injury, IRI) is the most common cause of AKI. Adiponectin (APN) has the effect of anti ischemia-reperfusion injury[2-5], and plays an important role in the development of renal fibrosis in UUO model[6]. PH domain and leucine zipper containing 1APPL1) is a signal transduction protein specifically binding to APN receptor on APN signal pathway. Recently, it has been proved that it is abundantly expressed and plays a protective role in the fibrosis process of heart, liver and other organs[7,8], and it has been found that the up regulation of APPL1 may be involved in the process of renal fibrosis in renal IRI mice[9], some studies have shown that protein kinase B Protein kinase B (PKB/Akt) is involved in acute renal injury. And the process of chronic fibrosis[10], and there are also a large number of experimental tips APPL1 is involved in cell regulation through Akt pathway[11]. Is it involved in the overexpression of fibrosis in the later stage of AKI mediated by Akt Cheng did not study to observe APPL1 mediated by Akt Protective effect on renal fibrosis in mice with renal IRI.

2. Materials and methods

2.1. Grouping

Approved by animal ethics center of Foshan first people's Hospital (batch No.: 20180135). The experimental animals were provided by the Clinical Research Institute of the first people's Hospital of Foshan, and all mice aged 8 weeks and weighing 20 ~ 25 g were selected. Twelve male C57BL / 6 mice were randomly divided into two groups with 6 mice in each group: sham operation group (WT-C group) and renal ischemia-reperfusion group (WT-I/R group); 48 APPL1 knockout mice were randomly divided into 4 groups with 12 mice in each group: APPL1 knockout sham operation group (AK-C group), APPL1 knockout renal ischemia-reperfusion group (AK-I / R group), APPL1 knockout + Akt inhibitor treatment group (AK-L group) and APPL1 knockout + normal saline treatment group (AK-S group)

2.2. Modeling and administration method

Except for WT-C group and AK-C group, the other groups prepared the renal IRI model by clamping the renal pedicle on both sides for 30min and restoring renal perfusion according to the literature[12]: The mice fasted for 12h before the experiment, drank water freely, and were anesthetized by intraperitoneal injection of 0.003ml/g10% chloral hydrate. After successful anesthesia, the abdominal cavity was opened along the abdominal midline to expose the left and right renal pedicle of the mice, and the bilateral renal pedicle was clamped at the same time with a non-invasive artery clamp under the opening of the left renal artery and above the opening of the right renal artery. The kidney changes from bright red to purple black, indicating successful ischemia, and then the abdominal cavity is closed layer by layer. After blocking the left and right renal pedicle for 30min, open the abdominal cavity, loosen the arterial clamp and restore the renal blood flow. After the bilateral kidneys change from purple black to pink, it indicates that the

reperfusion is successful, and finally close the abdominal cavity WT-C group and AK-C group only separated bilateral renal arteries without clamping The AK-L group was given LY294002 (adooq 40296) 6mg/kg intraperitoneally one minute after the successful preparation of the mouse bilateral kidney IRI model, and then the same dose of LY294002 was injected intraperitoneally every 24 hours to the seventh day. The AK-S group was injected intraperitoneally with the same dose of normal saline at the same time point

2.3. Data collection

1. Detection of renal function indexes at 24h after reperfusion, 6 mice in each group were randomly taken. After intraperitoneal injection of chloral hydrate anesthesia, the blood samples of orbital medial canthus venous plexus were centrifuged for 10min, and the upper serum was taken. The concentrations of bun and SCR in serum were detected by AD-VIA1800 automatic biochemical analyzer (Siemens, Germany)

2. Renal tubular injury score: Six mice randomly selected from each group at 24h after reperfusion were anesthetized by intraperitoneal injection of chloral hydrate. The left kidney was cut along the longitudinal axis coronal plane, fixed in 10% neutral formaldehyde solution and embedded in paraffin μm section, and observe the renal tubular necrosis under the optical microscope after he staining 20 visual fields were randomly selected, and the same researcher used the semi quantitative pathological evaluation method to score the renal tubular injury according to the degree of renal tubular necrosis[13]: If there is no abnormality in the renal tubular, it is 0< 5% of renal tubular necrosis was 1 point[5%, 25%] of renal tubular necrosis was 2 points[25%, 75%] of renal tubular necrosis is 3 points; more than 75% of renal tubular necrosis is 4 points

The kidneys of 3 mice in each group were taken from the left side of the abdominal cavity and perfused with chloral for 14 days. Take the renal cortex tissue to prepare paraffin sections, conventional dewaxing and rehydration, Sirius red saturated picric acid (Beijing solebao Technology Co., Ltd.) staining for 30min, direct differentiation and dehydration with absolute alcohol, and seal with xylene transparent neutral gum. Two slices were randomly selected, and five cortical fields were selected for each slice（× 400), with red collagen deposition as the positive signal. Imageproplus multimedia color pathological image analysis software is used to analyze and measure the ratio of collagen deposition area of renal interstitium to the total area of renal tubulointerstitium, and take the average value to reflect the degree of renal fibrosis.

4. Detection of renal fibrosis index at 14 days after reperfusion, renal collagen 1 (COL1), fibronectin (FN) and α- Smooth actin（α- Expression level of SMA). Take kidney tissue and prepare 5 μm thick frozen sections, fixed and sealed, respectively added with corresponding primary antibody and fluorescent. Alexa-488 labeled secondary antibody shaking table for incubation, and sealed with DAPI sealant. Five visual fields were selected at the junction of skin and marrow of renal tissue in each slice, and observed

and photographed under confocal microscope (Ni Kon company, Japan) NIS elements Br 3.0 software was used for analysis to calculate the percentage of fluorescence positive area in the total visual field area and reflect the expression level of the target protein COL1, FN and in renal tissue were measured by Western blot α- Expression level of SMA. The kidney tissue was centrifuged and homogenized to determine the protein concentration, and 50 μg protein samples were separated by SDS-PAGE electrophoresis, and semi dry electricity was transferred to PVDF membrane. After sealing and washing the film, add the corresponding primary antibody and horseradish peroxidase labeled secondary antibody respectively according to the steps. After incubation in a shaking table, add a chromogenic substrate in the dark room for chromogenic exposure and scanning. Image-j image analysis system is used for analysis, GAPDH is used as the internal parameter, and the ratio of the integrated optical density of the target protein band to the integrated optical density of GAPDH band reflects the expression level of the target protein.

5. Detection of renal APPL1 and phosphorylated. Akt expression. At 24h and 14d after reperfusion, the expression of APPL1 and the concentration of phosphorylated Akt (p-Akt) in renal tissue were measured by Western blot Take kidney tissue homogenate, add corresponding primary antibody and horseradish peroxidase labeled secondary antibody, and shake and incubate at room temperature Image-j image analysis system is used for analysis, GAPDH is used as the internal parameter, and the ratio of the integrated optical density of the target protein band to the integrated optical density of GAPDH band reflects the expression level of the target protein.

4. Statistical processing: Adopt spss16 0 software for analysis, the measurement data of normal distribution $\bar{x} \pm s$ are expressed in, and the comparison between groups adopts one-way ANOVA $P < 0.05$ means the difference is statistically significant

3. Result

3.1. Comparison of serum BUN and SCR concentrations and renal tubular injury scores at 24 hours after reperfusion

There was no significant difference between WT-C group and AK-C group ($P > 0.05$). There was no significant difference between AK-I/R group and AK-S group ($P>0.05$). Compared with WT-C group and AK-C group, the concentration and renal fraction of serum BUN and SCR in WT-I/R group, AK-I/R group, AK-L group and AK-S group were significantly higher at 24h reperfusion ($P<0.05$). Compared with WT-I/R group, AK-I/R group, AK-L group, AK-S group ($P<0.05$), AK-L group was the highest ($P<0.05$) (**Table 1**)

Table 1. 6 concentration of serum BUN and SCR and score of renal tubular injury of mice in group 24 after reperfusion

Group bun (mg / L)	BUN(mg/L)	Scr(mg/L)	Renal tubular injury score (score)
WT-C group (n=6)	94±13	8.4±1.4	0
WT-I / R group (n=6)	368±51[a]	34.4±2.3[a]	2.62±0.21[a]
AK-C group (n=6)	102±16	9.6±1.5	0

AK-I / R group (n=6)	492±62[ab]	48.3±2.1[ab]	3.12±0.25[ab]
AK-L group (n=6)	686±58[abe]	67.5±2.6[abc]	3.73±0.26[abc]
AK-S group (n=6)	485±61[ab]	49.1±2.7[ab]	3.07±0.24[ab]

Note: Compared with WT-C group or AK-C group, AP<0.05. Compared with WT-I / R group, BP<0.05. Compared with other groups, CP<0.05

3.2. Comparison of renal fibrosis after 14 days of reperfusion

There was no significant difference between renal reperfusion group and renal reperfusion group (P; AK-I/R group, AK-S group (P>0.5). Compared with WT-C group, AK-C group, WT-I/R group, AK-I/R group, AK-L group and AK-S group, the degree of renal fibrosis increased significantly (P<0.05). Compared with WT-I/R group, AK-I/R group, AK-L group and AK-S group increased significantly (P<0.05), and AK-L group increased significantly (P<0.05) (**Table 2**)

Table 2. Comparison of renal fibrosis degree of mice in group 2 at 14 days of reperfusion

group	Degree of renal fibrosis (%)
WT-C group (n=6)	0.28±0.04
WT-I / R group (n=6)	6.14±0.67[a]
AK-C group (n=6)	0.35±0.05
AK-I / R group (n=6)	7.84±0.46[ab]
AK-L group (n=6)	8.76±0.59[abc]
AK-S group (n=6)	7.77±0.56[ab]

Note: Compared with WT-C group or AK-C group, [a]P<0.05. Compared with WT-I/R group, [b]P<0.05. Compared with other groups, [c]P<0.05

3.3. At 14 days of reperfusion, COL1, FN and α-Comparison of SMA expression.

COL1, FN and α-. There was no significant difference in the expression of SMA between WT-C group and AK-C group (P>0.5). There was no significant difference between AK-I/R group and AK-S group (P>0.05). Compared with WT-C group and AK-C group, the eggs of WT-I/R group, AK-I/R group, AK-L group and AK-S group increased (P<0.05). Compared with WT-I/R group, the protein expression of AK-I/R group, AK-L group and AK-S group increased significantly (P<0.05), and AK-L group was the highest (P<0.05) (**Table 3**)

3.4. Comparison of APPL1 expression and Akt phosphorylation in renal tissue

At 24h and 14d after reperfusion, compared with WT-C group, the expression of APPL1 and Akt phosphorylation were significantly increased in WT-I/R group (P<0.05). At 24h after reperfusion, compared with AK-C group, Akt phosphorylation level in AK-I/R group and AK-S group increased significantly (P<0.05), and Akt phosphorylation level in AK-L group decreased significantly (P<0.05) compared with AK-I/R group (**Table 4**)

Table 3. 6 expression of COL1, FN and mRNA in renal tissue of mice in group 36 at 14 days of reperfusion α- Comparison of SMA expression levels

group	COL1		FN		α- SMA	
	Western blot method	Immunofluorescence method	Western blot method	Immunofluorescence method	Western blot method	Immunofluorescence method

WT-C group (n=6)	0.087±0.015	1.72±0.19	0.183±0.017	1.99±0.21	0.072±0.012	1.02±0.09
WT-I / R group (n=6)	0.842±0.073[a]	14.46±2.11[a]	0.809±0.093[a]	15.05±2.53[a]	0.453±0.034[a]	8.32±1.78[a]
AK-C group (n=6)	0.092±0.014	1.70±0.17	0.178±0.016	1.87±0.23	0.076±0.012	1.11±0.10
AK-I / R group (n=6)	0.963±0.068[ab]	15.92±2.09[ab]	0.988±0.096[ab]	16.25±2.68[ab]	0.575±0.035[ab]	9.82±1.94[ab]
AK-L group (n=6)	1.315±0.075[abc]	17.13±2.20[abc]	1.289±0.102[abc]	18.32±2.24[abc]	0.789±0.032[abc]	11.64±1.86[abc]
AK-S group (n=6)	1.002±0.069[ab]	16.28±2.04[ab]	0.962±0.110[ab]	16.46±2.59[ab]	0.583±0.038[ab]	9.79±2.12[b]

Note: Compared with WT-C group or AK-C group, [a]$P < 0.05$. Compared with WT-I/R group, [b]$P<0.05$. Compared with other groups, [c]$P < 0.05$.

Table 4. Comparison of APPL1 expression and Akt phosphorylation level in renal tissue of mice in 6 groups at 24h and 14d of reperfusion

group	24h reperfusion		Reperfusion for 14 days	
	APPL1	p-Akt	APPL1	p-Akt
WT-C group (n=6)	0.193±0.013	1.12±0.31	0.172±0.012	1.02±0.25
WT-I / R group (n=6)	1.085±0.225[a]	1.85±0.29[a]	0.835±0.110[a]	1.54±0.30[a]
AK-C group (n=6)	-	1.16±0.31	-	1.06±0.25
AK-I / R group (n=6)	-	1.56±0.28[b]	-	1.25±0.27[b]
AK-L group (n=6)	-	1.20±0.24[c]	-	1.01±0.26[c]
AK-S group (n=6)	-	1.55±0.28[b]	-	1.26±0.23[b]

Note: Compared with WT-C group, [a]$P<0.05$. Compared with AK-C group, [b]$P<0.05$. Compared with AK-I/R group, [c]$P<0.05$.

4. Discuss

The incidence rate of CKD is increasing year by year. If it fails to be treated in time, it will eventually progress to end-stage renal disease (ESRD). CKD is one of the most common and can be developed in critical patients IRI is one of the most common causes of AKI. Studies have shown that renal IRI can directly damage glomerular capillary endothelial cells and stimulate local inflammatory response. Various inflammatory factors produced locally in the kidney can activate fibroblasts, make them proliferate and apoptosis, and form a large number of fibrous scar tissue. Renal IRI often occurs during perioperative period. To explore the mechanism of fibrosis in the later stage of renal. Iri is of great significance to clarify the correlation between AKI and CKD and its pathophysiological mechanism.

In this study, the IRI model of mouse kidney was prepared according to the method of reference[12]. The results showed that compared with WT-C group and AK-C group, the concentrations of blood bun and SCR and the score of renal tubular injury in WT-I/R group, AK-I/R group, AK-L group and AK-S group increased significantly ($P<0.05$), indicating that the mouse renal IRI model was successfully prepared.

Previous studies of our research group have proved that APN signaling pathway is involved in the regulation of renal

fibrosis[14], and APN can reduce the fibrosis caused by IRI and AKI in mouse kidney[15]. APPL1 is a signal transduction protein that specifically binds to APN receptor on APN signal pathway and participates in the regulation of multiple signal pathways. At the same time, ap-pl1 can also interact with a variety of receptors and intracellular signal proteins through Akt domain in a dependent or independent phosphorylated lysine binding pathway, and play an important role in cell survival, proliferation, differentiation and apoptosis[16]. In this study, ordinary mice and APPL1 knockout mice were used to prepare renal IRI models respectively, and one group of APPL1 knockout mouse models was treated with Akt inhibitor LY294002 to block Akt activation. The expression changes of APPL1 and p-Akt in the pathological process and the degree of renal fibrosis in mice in the middle and late stage were observed. Studies have confirmed that significant renal fibrosis can occur 14 days after AKI[17], so this study selected the time point 14 days after IRI to measure the degree of renal fibrosis. In addition to the direct observation of renal fibrosis by Sirius red picric acid staining, COL-1 and COL-1 in renal tissue were measured by Western blot and immunofluorescence, respectively α-. The expression levels of SMA and FN were measured to further observe the situation of renal fibrosis.

It can be seen from the results that 24 hours after IRI, compared with WT-C group and AK-C group, the concentrations of blood bun and SCR and the score of renal tubular injury in WT-I/R group, AK-I/R group, AK-L group and AK-S group were significantly increased, indicating that the kidneys of mice with surgical models in each group were significantly acute injured. Among them, the damage in AK-I/R group is more obvious than that in WT-I/R group, and the damage in AK-L group is the most serious 14 days after the occurrence of renal IRI in mice, the score of renal fibrosis in WT-I/R group, AK-I/R group, AK-L group and AK-S group increased, and COL-1 and α-. The expression of SMA and FN is up-regulated, and the fibrosis trend is consistent with the early injury trend, indicating that the early acute renal tubular injury leads to the later renal fibrosis and affects the severity of renal fibrosis. The acute injury and chronic fibrosis in AK-I/R group were more serious than those in WT-I/R group, suggesting that APPL1 has a protective effect on acute injury and chronic fibrosis of renal IRI in mice.

The results of the expression of APPL1 and p-Akt in renal tissue of each group detected at 24 hours and 14 days after the occurrence of IRI in mice kidney showed that the expression of APPL1 and p-Akt in WT-I/R group was significantly higher than that in WT-C group ($P<0.05$), the expression of Akt in renal tissue of AK-I/R group and AK-S group was lower than that in WT-I/R group, and there was no significant difference in the expression of p-Akt between AK-L group and AK-C group after treatment with Akt inhibitor ($P>0.05$). The degree of renal fibrosis in AK-L group is the most serious, and there is no significant difference between AK-I/R group and AK-S group, suggesting that the expression of p-Akt has a protective effect on chronic fibrosis in mice.

The results of this study suggest that the expression of APPL1 increases after mouse renal IRI, which can enhance Akt phosphorylation. P-Akt expression activates the downstream pathway to regulate the apoptosis of renal tubular epithelial cells, so as to protect mouse renal IRI and chronic fibrosis After renal IRI, APPL1 gene knockout mice had no APPL1 expression, p-Akt expression was limited, and the degree of chronic renal fibrosis increased. After APPL1 knockout mice were treated with Akt inhibitor, the expression of p-Akt was inhibited, and the degree of chronic renal fibrosis was more serious. There was no significant difference in the expression of p-Akt between AK-I/R group and AK-S group. It is proved that APPL1 has a protective effect on renal IRI and chronic fibrosis in mice by regulating Akt. The mechanism may be to enhance the expression of APPL1, increase the phosphorylation activation of Akt, and then produce a series of cell regulation, so as to reduce the acute injury of renal IRI and chronic fibrosis in later stage.

The deficiency of this study is that it failed to block the expression of APPL1 after IRI in WT mice, and further observe the protective effect of APPL1 expression on renal fibrosis after IRI in mice, which needs to be further improved by follow-up research.

In conclusion, Akt mediated APPL1 has a protective effect on renal fibrosis in mice with renal IRI How to use appropriate drugs and methods in clinic to improve the expression of APPL1 or p-Akt and enhance the protective effect of chronic fibrosis after renal IRI will be the direction of future research.

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