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Oleanolic Acid Improves the Symptom of Renal Ischemia Reperfusion Injury via the PI3K/AKT Pathway

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Keywords
Oleanolic acid · LY294002 · PI3K/AKT signal pathway · PDK1 · Renal ischemia

Abstract
Purpose: The aim of this study was to investigate the therapeutic effect of oleanolic acid (OA) on the renal ischemia reperfusion injury (RIRI) and the possible mechanism. ***Methods:*** The RIRI model was successfully established in rats. OA, LY294002 (a PI3K inhibitor), and OA combined with LY294002 were dosed to rats in 3 therapeutic groups, respectively. The blood was collected to detect the concentration of Cr and BUN by ELISA. The kidney of each rat was collected to detect the concentration of renal injury factor (Kim-1) and the HE staining was performed. Western blot was used to detect the expression level of PI3K, p-AKT, AKT, PDK1, Skp2, and p27 in the renal tissue homogenate. ***Results:*** The symptom of vacuolar degeneration and interstitial edema was greatly improved in the rat kidney from the 3 therapeutic groups, compared with that from the RIRI model group. No significant difference was observed among the 3 therapeutic groups. The concentration of Cr in the 3 therapeutic groups was greatly lower than that in the RIRI model group. The expression level of p-AKT/AKT, PI3K, PDK1, Skp2, and p27 in OA group, LY294002 group, and OA combined with LY294002 group was significantly lower than that in the RIRI model group, respectively. ***Conclusion:*** OA could improve the symptom of RIRI, possibly by inhibiting PI3K/AKT signal pathway.

Introduction

Kidney is highly sensitive to ischemia. The symptom of tissue injury may not be improved or even get worse after kidney recovers from ischemia, which is called renal ischemia reperfusion injury (RIRI) [1]. RIRI can be induced by renal vascular surgery, kidney transplantation, cardiac arrest, or hypotensive shock. Currently, the incidence and mortality of RIRI have increased greatly as more and more kidney transplantation, microcirculation recanalization after shock, and major cardiac surgeries were performed in patients [2, 3]. Therefore, prevention and therapeutic methods for RIRI are urgent for clinic.

PI3K/AKT signal pathway is one of the central signal pathways that regulate cell growth. It has been reported

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that the regulation function of PI3K/AKT signal pathway was observed in the research of central nervous system ischemia-reperfusion injury, cardiac ischemia reperfusion injury, and RIRI, which mainly includes the function of anti-apoptotic and reducing oxidative stress by activating the PI3K/AKT signal pathway [4–7]. However, the symptom of ischemia reperfusion injury was also reported to be improved due to the autophagy reaction induced by the PI3K/AKT signal pathway [8, 9].

Oleanolic acid (OA) is a natural non-toxic triterpenoid, which has liver protection and antiviral function. OA is also used for cosmetics and health products [10, 11]. It is reported that the growth of gallbladder carcinoma cells could be inhibited due to the inactivation of PI3K/AKT/mTOR signal pathway induced by OA [12]. OA was also reported to reduce oxidative stress by activating PI3K/AKT/eNOS signal pathway within vascular endothelial cells [13]. However, the regulation function and mechanism of OA on RIRI were rarely investigated. In the present study, the therapeutic effects of OA on RIRI were investigated, as well as the impacts of OA on the PI3K/AKT signal pathway.

Materials and Methods

Experimental Groups

Thirty-six SD rats were purchased from Hunan SJA Laboratory Animal Co., Ltd. (SCXK2016-0002). Six groups were divided in the present study: (1) control; (2) sham (only renal capsule was destroyed when the surgery was performed.); (3) model (same volume of normal saline); (4) model with OA treatment (50 mg/kg/day for 14 days before surgery); (5) model with LY294002 treatment (working concentration: 40 μmol/mL [14], dosage: 40 μL/day for 14 days before surgery); and (6) model with OA and LY294002 treatment (the same dosage as group 4 and 5).

The Establishment of RIRI Model in Rats

After treatment for 14 days, the surgery for RIRI model was performed on each rat. 350 mg/kg 10% chloral hydrate was injected intraperitoneally to hocus rats. Rats were fixed and laparotomized. Lipids around the kidney were removed, and a tweezer was located across the blood vessels beneath the kidney. A length of twine was guided by the tweezer to fasten the renal vessel to make the kidney under ischemia. The change of renal color was observed to determine if kidney was under ischemia. After ischemia for 45 min, the twine was removed to induce renal reperfusion and the color of kidney was recovered to ruddy. Subsequently, the surgical incision was sutured and smeared with iodine. Rats were located on the heating pad to make them revive quickly.

HE Staining

Two days after the surgery, kidneys of each rat were collected and washed over by sterile water for a couple of hours. The tissue was dehydrated by 70, 80, and 90% ethanol solution successively and mixed with equal quality of ethanol and xylene. After 15 min incubation, the tissue was mixed with equal quality of xylene for 15 min. Repeat the step until the tissue looked transparent. Subsequently, the tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Pictures were taken by an inverted microscope (Olympus).

Enzyme-Linked Immunosorbent Assay

According to the instruction of the manufacturer (Sigma), the concentration of Cr and BUN was detected in the blood of each rat and the concentration of Kim-1 was detected in the kidney. Basically, the operation includes sample adding, enzyme adding, incubation, working solution preparing, washing, dyeing, terminating, and detecting. Linear regression equation was described based on the concentration of standards and the OD value. The concentrations of the samples were calculated according to the equation, detected OD value, and dilution factor.

Western Blot Assay

The kidneys were isolated from the rats. Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) were used to isolate the proteins from the tissues. Approximately 30 μg of protein was separated on 12% SDS-polyacrylamide gel. The gel was transferred to PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in TBST (Trisbufferedsaline/0.1% Tween-20, pH 7.4) for 1 h at room temperature and incubated overnight with primary rabbit anti-human antibodies to PI3K (1:1,000), p-AKT (1:1,000), AKT (1:1,000), PDK1 (1:1,000), Skp2 (1:1,000), p27 (1:1,000), and β-actin (1:1,000) (Abcam, Burlingame, CA, USA). A horseradish peroxidase-conjugated antibody against rabbit IgG (1:5,000, Abcam, Burlingame, CA, USA) was used as a secondary antibody. Blots were incubated with the ECL reagents (Beyetime, Jiangsu Province, China) and exposed to Tanon 5200-multi to detect protein expression. Three independent assays were performed. Quantity One software was used to calculate the gray value.

Statistical Analysis

Statistically significant differences for continuous variables were determined using a one-way ANOVA with the least significant difference (LSD) test for the normal distribution data. All testing was performed using GraphPad Prism 6 software. A difference with a p value of <0.05 was considered to be significant.

Results

Therapeutic Effect of OA and LY294002 Was Achieved on RIRI

In order to investigate the effects of OA on RIRI, HE staining and ELISA were performed on the kidney or blood of each rat. As shown in Figure 1, compared with the control and sham groups, vacuolar degeneration, coagulative necrosis, and exfoliation were observed in the renal tubular epithelial cells in the model group, as well as tube-like materials involved in renal tubules and interstitial edema. The symptom of vacuolar degeneration and interstitial edema was improved greatly in the model with

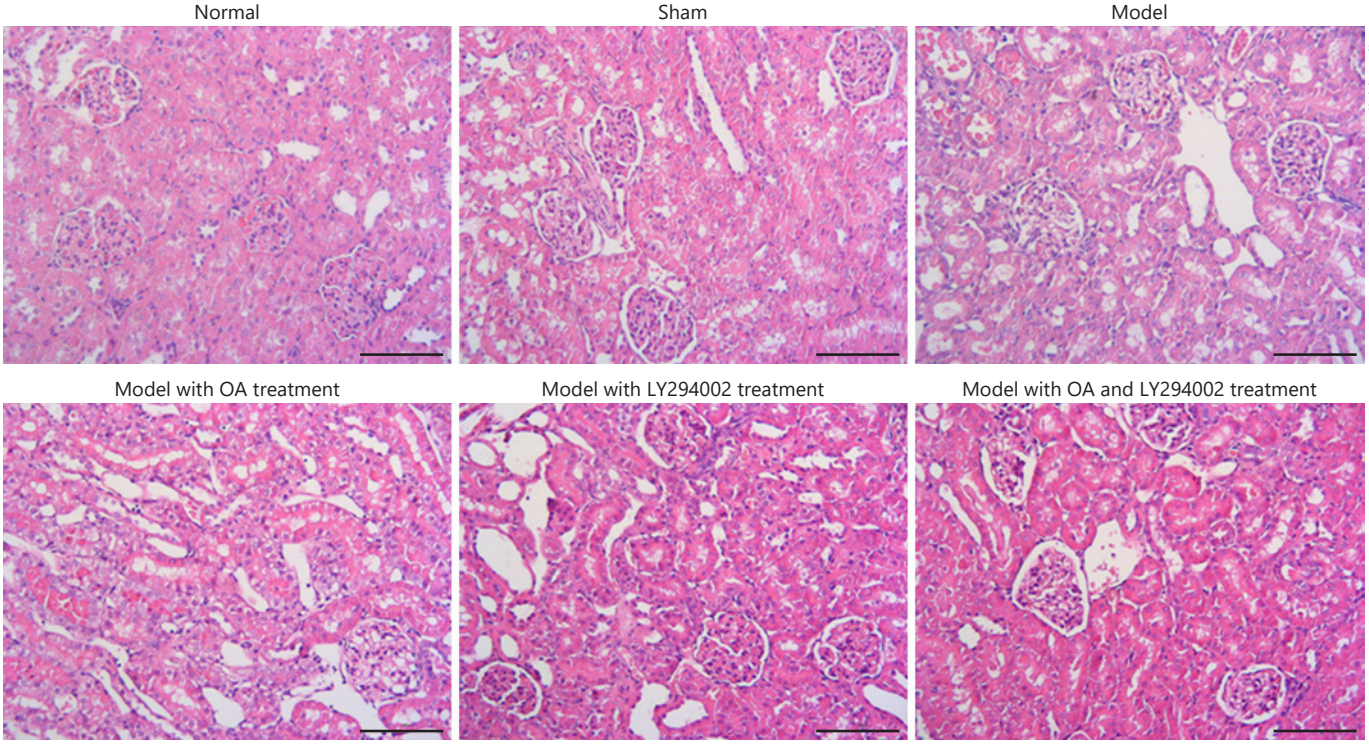

Fig. 1. Results of HE staining on kidney. Scale bar, ×200 (n = 6).

OA treatment group, model with LY294002 treatment group, and model with OA and LY294002 treatment group, with no obvious difference among the 3 groups.

The concentrations of Cr, BUN, and Kim-1 detected by ELISA are shown in Figure 2. The concentration of Cr was greatly increased in the rats from the model group compared with that from the sham group ($p < 0.05$). However, there were no differences in the concentration of Kim-1 and BUN between the model group and sham group. The concentration of Cr in rats' blood from the 3 therapeutic groups was significantly lower than that in the model group ($p < 0.05$). The concentration of BUN in rats' blood from the OA treatment group and combination group was greatly lower than that in the model group ($p < 0.05$). Taken together, these results indicated that treatments with OA or LY294002 could improve the symptom of RIRI.

Effects of OA or LY294002 on the PI3K/AKT Signal Pathway

In order to explore the effects of OA on the PI3K/AKT signal pathway, Western blot was performed to detect the expression level of related proteins in the kidney of each rat. The results of Western blot are shown in Figure 3. The expression level of p-AKT/AKT and PI3K in the kidney of rats from the model group was significantly higher than that from the sham group ($p < 0.05$). In contrast, p27, PDK1, and Skp2 were expressed in lower level ($p < 0.05$). Compared with the model group, p-AKT/AKT and PI3K were expressed in lower level in the kidney of rats from the 3 therapeutic groups ($p < 0.05$). Meanwhile, the expression level of p27, PDK1, and Skp2 in the kidney of rats from the 3 therapeutic groups was significantly higher than that from the model group ($p < 0.05$). Interestingly, the expression level of PDK1 and Skp2 was increased more strikingly in the combinational treatment groups than individual ingredient treatment. The results were not observed in the expression of p-AKT/AKT, PI3K, and p27.

Discussion

Cr is the metabolite of creatine, which is one of the energy providers for muscles. The concentration of Cr in blood will increase when renal injury happens. Carbamide is the final metabolite of proteins in our body, which indicates that the concentration of BUN in blood

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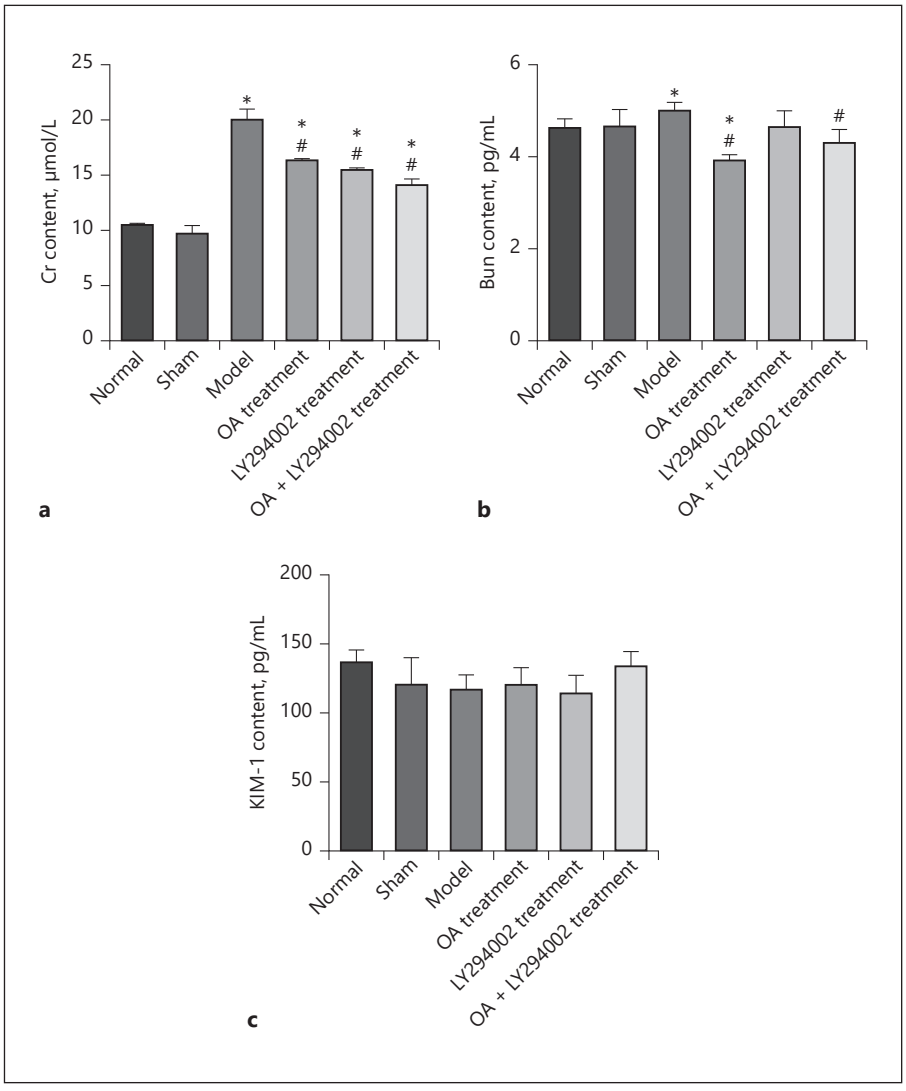

Fig. 2. Concentration of Cr, BUN, and Kim-1 was detected by ELISA. **a** The concentration of Cr in the blood of each rat. **b** Concentration of BUN in the blood of each rat. **c** Concentration of Kim-1 in the kidney of each rat ($^{*}p < 0.05$, vs. sham group; $^{\#}p < 0.05$, vs. model group).

could be impacted by the intake and metabolism level of proteins. It is also reported that the concentration of BUN in blood was related with the filtering effect of kidney [15]. Kim-1 was named as renal injury factor 1 as it is reported that the expression level of Kim-1 in kidney increased significantly after the renal injury happened [16]. In the present study, in addition to HE staining on the kidney slides, concentration detection of Cr and BUN in blood as well as concentration detection of Kim-1 in kidney was used to evaluate the degree of renal injure. The results of HE staining indicated that the symptom of RIRI was improved greatly by all the 3 therapeutic treatments. The results of ELISA showed that the concentration of Cr and BUN was significantly changed in the RIRI model established in the present study, not Kim-1, which indicated that the renal injury brought by surgery was limited. In addition, OA treatment could decrease the concentration of BUN in blood, which is consistent with the result of HE staining. However, these results might also be caused by the decrease of metabolic level.

PI3K/AKT signal pathway was reported to be related to autophagy reaction in RIRI model [8, 9]. In PI3K/AKT signal pathway, the biological signal transfer between PI3K and AKT is regulated by protein PDK1. The expression of p27 is regulated by AKT, and p27 could exert cell growth inhibition function. Skp2 expression level is also regulated by AKT, which mediates the ubiquitination degradation pathway for p27 [17–19]. In the present study, in additional to AKT, p-AKT, and PI3K,

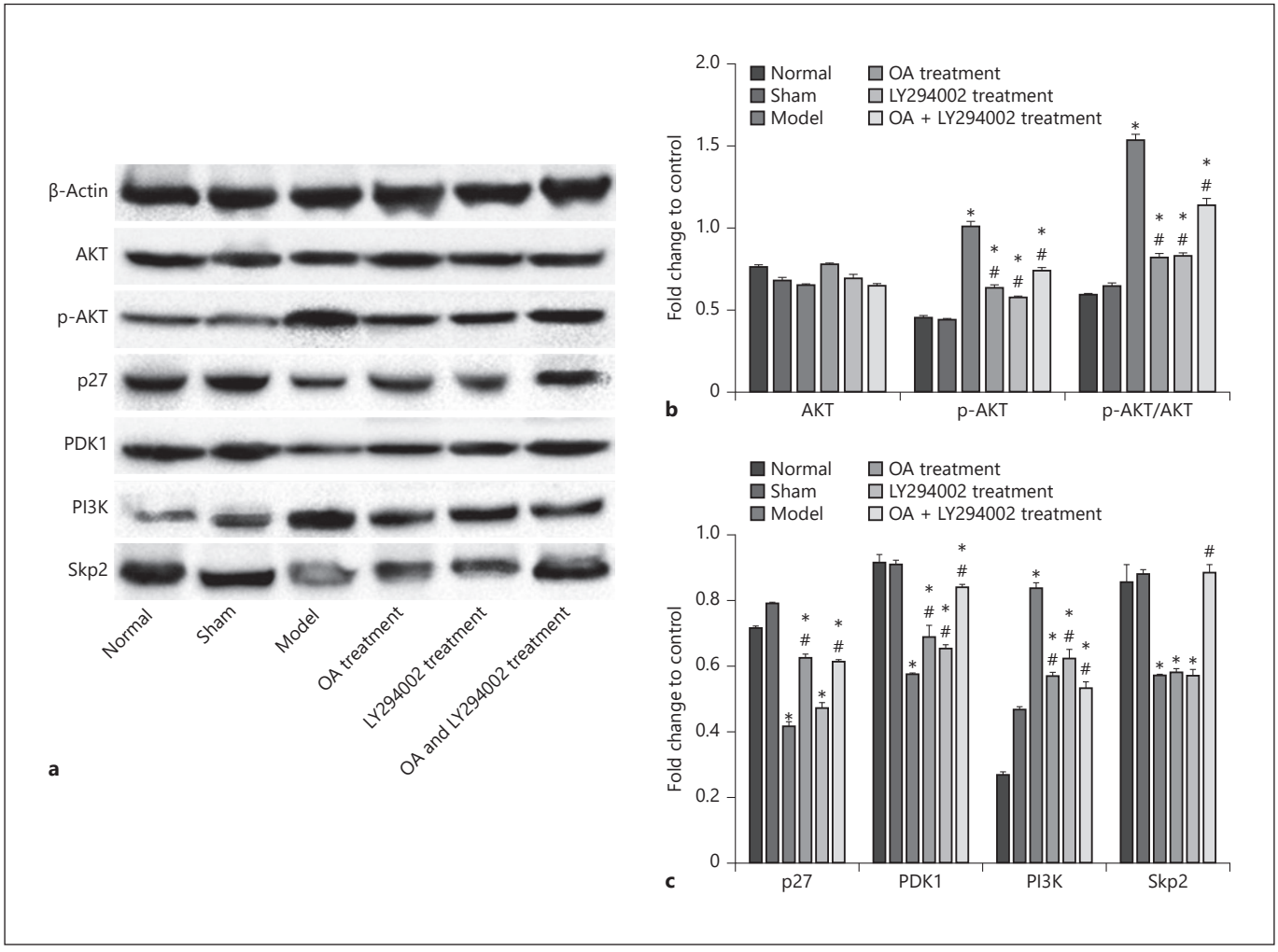

Fig. 3. Expression level of PI3K, p-AKT, AKT, PDK1, Skp2, and p27 was detected by Western blot. **a** Pictures of blots for each protein. **b** Gray values of p-AKT/AKT calculated by Quantity One. **c** Gray values of p27, PDK1, PI3K, and Skp2 calculated by Quantity One (*$p < 0.05$, vs. sham group; #$p < 0.05$, vs. model group).

the expression level of p27, PDK1, and Skp2 was also detected. P-AKT/AKT and PI3K were found up-regulated in the kidney from the RIRI model. OA treatment could reverse the regulation. These results not only indicated that OA could inhibit PI3K/AKT signal pathway but also showed that the function of PI3K/AKT signal pathway was closely related with RIRI symptom. It is interesting that the expression level of PDK1 and Skp2 was increased greater in combinational treatment groups than individual ingredient treatment, not for the expression of p-AKT/AKT and PI3K. The difference, as well as the relationship between autophagy reaction and RIRI improvement treated by OA, will be deeply investigated in future works.

Statement of Ethics

We declare that all animal experiments involved in this manuscript were authorized by the Ethical Committee of Jiangxi Provincial People's Hospital and carried out according to the guidelines for care and use of laboratory animals as well as to the principles of laboratory animal care and protection. And the research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors declare there is no conflict of interest regarding the publication of this paper.

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Author Contributions

JinRan Yang: the major operator of the study. Xinchang Li and Hua Yang: writing of the paper. Chengmei Long: design of the study.

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