Renoprotective Effect of Oridonin in a Mouse Model of Acute Kidney Injury via Suppression of Macrophage Involved Inflammation

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Ischemia–reperfusion injury (IRI) is the major cause of acute kidney injury (AKI). The previous studies demonstrated that Oridonin can protect kidney against IRI-induced AKI, but the underlying molecular mechanism is unclear. In this study, it showed that Oridonin significantly improved kidney damage, and inhibited the expression of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and MCP-1, as well as macrophage marker F4/80 in kidney and the secretion of inflammatory cytokins in serum of AKI mice in vivo. In addition, Oridonin also effectively reduced the expression and secretion of lipopolysaccharide (LPS)-induced inflammatory factors in macrophage cell line RAW264.7 in vitro. Notably, Oridonin strongly downregulated Mincle and AKT/nuclear factor-kappaB (NF-κB) signaling both in vivo and in vitro, and the results of cellular recovery experiments of overexpression of Mincle in macrophage suggested that Oridonin suppressed inflammatory response of macrophage through inhibiting Mincle, which may be the underlying mechanism of Oridonin improving injury in kidney of AKI mice. In summary, the above results indicated that Oridonin can protect kidney from IRI-induced inflammation and injury by inhibiting the expression of Mincle in macrophage.

Key words Oridonin; acute kidney injury (AKI); macrophage; mincle; inflammation

INTRODUCTION

An important feature of acute kidney injury (AKI) is the sudden decline in kidney function. It is a global health issue that has aroused widespread concern in society. According to statistics, about 13.3 million people worldwide suffer from AKI every year, 85% of whom are in developing countries, which will kill 1.7 million people.1,2 Notably, AKI is not a single disease, and it may be a complication of multiple diseases. Normally, it may be caused by sepsis, shock, ischemia–reperfusion, and poisoning. After long-term development, this disease tends to progress to chronic kidney disease, congestive heart failure and end-stage renal disease.3–5) In summary, AKI can cause a huge economic burden, and it will virtually increase the psychological and physical burden on patients.

Renal ischemia–reperfusion is currently considered to be the most common cause of AKI, and it usually occurs during trauma, surgical resection, or kidney transplantation. Macrophages are one of the key inflammatory cells that play an important role in AKI progression. Mincle is mainly expressed on myeloid cells and neutrophils, especially on macrophages. It has a highly retained C-type lectin domain which is a major player in antifungal immunity. Mincle were originally identified by Matsumoto as a lipopolysaccharide (LPS)-induced inflammatory factors in macrophage cell line RAW264.7 via the Card9-Bel10-MALT1 signaling, resulting in the formation of an effective T helper 1 (Th1) and Th17 subtypes which promote the expression of inflammatory factors to connect innate and adaptive immune responses.6) Recent researches have found that Mincle can also recognize all kinds of abnormal substances in cells, sense damage-related signals, and then respond to relevant responses. For instance, Mincle can bind to β-GlcCer which is a ubiquitous intracellular metabolite from damaged cells, activating and inducing cytokines, and triggering antigen-specific T cell responses.7) Mincle is a receptor that senses heterogeneous cell death, can recognize the endogenous ligand SAP130 released by dead cells, induc the production of inflammatory cytokines, and drive neutrophil infiltration into damaged tissues.8,9) Notably, Lan’s team confirm that Mincle is specifically induced on M1 macrophages and it plays a key role on maintaining the inflammation phenotype of M1 macrophages.10) Therefore, targeted mincle therapy may be the key to treating AKI disease.

Oridonin is a tetracyclic dipeptide natural compound with kauriene skeleton extracted from oridon, which has a wide range of biological functions, including anti-microbial, anti-tumor, anti-inflammatory, and anti-fibrosis. Many researches have reported the anti-tumor potential and mechanism of this natural diterpenoids in a series of cancer cell lines.10,11) and it has been confirmed that Oridonin as the anti-cancer medicine is currently undergoing phase I clinical trials in China. However, the role of Oridonin in kidney disease is rarely reported. Several researches have verified that Oridonin can reduce proteinuria and kidney damage in spontaneous lupus erythematosus mouse model, and in vitro studies have found that Oridonin can reduce the secretion of inflammatory cytokines and the inflammatory response induced by LPS after treatment with Oridonin.12,13)
In this study, we focused on revealing the potential of Oridonin in the treatment of AKI, and whether its underlying mechanism is related to the inhibition of Mincle in macrophages. This study can provide strong evidence for the screening of AKI therapeutics.

MATERIALS AND METHODS

Animals

Thirty-six of male C57BL/6 mice (aged 8–10 weeks) were obtained from Beijing Vital River Laboratory Animal Technologies Co., Ltd. (Beijing, China), and housed in temperature-controlled and humidity-controlled room of Animal Experiment Center of Southwest Medical University (21.0 ± 2.0°C and 65 ± 5%, respectively). All mice were stochastically divided into three groups: including Sham group, ischemia–reperfusion injury (IRI) model group (IRI group) and Oridonin treated IRI group (OR group) (15 mg/kg). Before surgery, mice were anesthetized with intraperitoneal injection of saline-diluted pentobarbital sodium (200 mg/kg). The AKI mouse model was established by clamping bilateral renal arteries for 35 min. Mice in sham group were only exposed abdominal cavity for 1 h. The cell survival rate was calculated according to the ab- sorbance value of each well.

Assessment of Renal Function

The level of serum creatinine and urea nitrogen were measured by using the appropriate kit (Jiancheng, Nanjing, China). Values are expressed as mmol/L of serum.

Hematoxylin–Eosin (H&E) and Periodic Acid–Schiff (PAS) Staining

The collected kidney tissue was placed in 4% paraformaldehyde for 24 h, and then placed in ethanol solutions of different concentrations, and placed in xylene and wax blocks for embedding. Before staining, the slides were put into a 60°C oven for 2 h, and then dewaxed and rehydrated. Some samples were stained with eosin and hematoxylin pigments, other samples add periodic acid alcohol and Schiff dye solution separately. Finally, Observed and photographed in a microscope camera.

Enzyme-Linked Immunosorbent Assay (ELISA)

The level of IL-1β, IL-6 and TNF-α in the cell culture supernatant were measured by using ELISA kits (IL-1β: Neobioscience, EMC001b; IL-6: Neobioscience, EMC004; TNF-α: Neobioscience, 500850). Perform sample testing according to the method provided by the reagent supplier, and calculate the concentration value of each group according to the standard curve.

Western Blot

All animal and cell sample were lysed in radio immunoprecipitation assay (RIPA) buffer on ice, and centrifuged to obtain the corresponding protein sample. Coomassie brilliant blue assay was used to determine the protein concentration of each sample, and the gel was run with a certain amount of protein samples. Each group of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to the polyvinylidene difluoride (PVDF) membrane. After incubating membrane with primary antibodies at 4°C overnight, the secondary antibodies were incubated at room temperature for 1h. The color of the band is developed with a color developing solution, and gray intensity of the band was calculated by Image J software.

Real-Time PCR

The total RNA was obtained according to the operating instructions of the Tiangen kit, and it was dissolved in enzyme-free water. According to the relevant instructions, we use reverse transcription kit to synthesize cDNA. RT-PCR reaction conditions were performed as follows: 37°C 15 min, 95°C 5 min, and then 40 cycles of

| Gene | Primer sequence (5’ to 3’) |
|------|----------------------------|
| IL-1β | S: TGCCACCTTTTGTACAGTGTG | A: AAGGTCACGGGAAGAACAC |
| IL-6 | S: AAGAAGTGGCAGAATGCATTCT | A: AAGTCGATCATGTTGTCATACA |
| TNF-α | S: CATCCTTCTCAAAATTTCCAGTGCACA | A: TGGGAGTAGAACAGGCTCAAACCC |
| MCP-1 | S: CTTCTGGGCGCTGGTCTCA | A: CCAGCCTACTCATTGGGTATCA |
| iNOS | S: GTTCTCAGCAGCAATACAGGAAGA | A: GTGGAACGGGTGACATGTCAC |
| F4/80 | S: TGGAGGTGGACAGAGCATAGTG | A: TTCATGTCGCTCAAGC |
| Mincle | S: ACCAAAATGGGCGTGATCCA | A: CATCTGGGGATTGAAAGCATC |
| GAPDH | S: AGGTGGGGTGAAACGGATTG | A: GGGGTCGTTGATGGCACA |
95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. The relative mRNA expression level of each gene was normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The sequences of primers are shown in Table 1.

**Immunofluorescence Staining** RAW264.7 cells were fixed in 4% paraformaldehyde, then blocking with bovine serum albumin at room temperature (r.t.) for 1 h, and incubated with the corresponding primary antibodies at 4 °C overnight. After washing with phosphate buffered saline (PBS) for 3 times, cells were incubated with secondary antibodies at r.t. for 1 h. The nucleus were stained by 4′,6-diamidino-2-phenylindole (DAPI). The immunofluorescence images were captured by Nikon Eclipse microscope (Nikon, Tokyo, Japan).

**Statistical Analysis** Statistical analysis results are presented as mean ± standard deviation (S.D.). Comparisons between groups were performed using one way ANOVA, \( p < 0.05 \) was considered significant. GraphPad Prism 7 software was used to calculate data for statistical results.

**RESULTS**

**Oridonin Inhibited Ischemia–Reperfusion Induced Renal Injury and Inflammation** In order to evaluate the protective effect of oridonin on AKI kidney induced by ischemia–reperfusion, we examined the blood creatinine, urea nitrogen and renal tubular injury index of mice in each group, as well as the expression of inflammatory cytokins in renal tissue and assessed the renal pathology by H&E and PAS staining. The result shown in the figure that the serum creatinine (Fig. 1a) and urea nitrogen (Fig. 1b) levels of the AKI model group were reduced when continuous injection of Oridonin for three days. Correspondingly, the tubular injury index demonstrated that Oridonin strongly reduce tubular necrosis in kidney of AKI mice (Fig. 1c). Real-time PCR results showed that Oridonin significantly down-regulated the mRNA levels of IL-1β, MCP-1 and F4/80 in kidney of AKI model group (Figs. 1d–f). Moreover, the results of pathological staining and immunofluorescence showed that Oridonin significantly reduced tubular necrosis (Fig. 1g) and protein level of inflammatory cytokins (Fig. 1h) in AKI mice, which effectively improved kidney damage, indicating that Oridonin had significant anti-injury and anti-inflammatory effects on AKI model.

**Oridonin Inhibited Mincle/AKT/NF-κB Signaling Pathway in Kidney of AKI Mice** To elucidate the therapeutic mechanism of Oridonin on renal inflammation, we further examined the activation of Mincle/AKT/NF-κB signaling after Oridonin administration. Western blot results showed that Oridonin intervention can significantly reduce the expression of inflammation-related proteins IL-1 and iNOS in AKI kidneys and decrease the activation of AKT and NF-κB pathway.
proteins (Figs. 2a–e), indicating that Oridonin can inhibit IRI-induced inflammation in kidney of AKI. Notably, Real-time PCR and Western blot results showed that Oridonin effectively reduced the mRNA and protein levels of Mincle in kidney of AKI model (Figs. 2f, g). In order to detect the location of Mincle in kidney, we performed immunofluorescence in each group (Fig. 2h), the results showed that Mincle was located in renal interstitium and co-localized with macrophage Marker F4/80, indicating that Mincle was mainly expressed in renal macrophages, and strongly up-regulated after AKI. Immuno-fluorescence results also showed that macrophage increased significantly after AKI, suggesting the important role of macrophage in the development of AKI. Notably, Oridonin significantly inhibited Mincle in renal macrophage of AKI mice. The above results indicate that Oridonin may improve kidney inflammation and injury by inhibiting Mincle/AKT/NF-κB signaling.

Oridonin Significantly Restored the RAW264.7 Macrophage Morphological Changes Induced by LPS In order to study the effect of Oridonin on inflammatory macrophages, we used LPS to stimulate RAW264.7 cells to construct an inflammatory cell model. The chemical structure of Oridonin is shown in the Fig. 3a. We therefore tested the toxicity of Oridonin at different concentrations on RAW264.7 cells. The CCK8 results show that when the concentration is 5 µM, Oridonin has no significant effect on cell morphology (Fig. 3b), which was used in subsequent in vitro experiments. We surprisingly found that Oridonin significantly improved LPS-induced macrophage morphology (Fig. 3c), including reducing intracellular vacuoles and making cells more solid.

Oridonin Suppressed the Secretion and Expression of Inflammatory Factors in LPS-Stimulated RAW264.7 Cells In order to clarify the anti-inflammatory effect of oridonin on LPS-stimulated RAW264.7 cells, we used real-time fluorescent PCR, ELISA, and immunofluorescence to measure the inflammatory factors secreted by each group of cells. The results presented that Oridonin significantly reduced the mRNA levels of IL-1β, IL-6, TNF-α, iNOS, and MCP-1 in LPS-stimulated cells (Figs. 4a–e). Furthermore, Oridonin also significantly down-regulated the secretion of IL-1β, IL-6 and TNF-α in supernatant in inflammatory cell model (Figs. 4f–h) as well as the protein levels of inflammatory cytokins in LPS-stimulated...
Macrophage (Fig. 4i). The above results show that Oridonin has a obvious anti-inflammatory effect in macrophage.

Oridonin Blocked the Mincle/AKT/NF-κB Signaling Pathway in Inflammatory Macrophage in Vitro

Mincle, as a pattern recognition receptor, is essential for promoting macrophage-related inflammation in kidney of AKI. The above animal experiment results showed that Oridonin can down-regulate the expression of Mincle in kidney of AKI. Therefore, we further investigated whether Oridonin can reduce the mRNA and protein level of Mincle in LPS-stimulated macrophage inflammatory model. The results of Western blot, immunofluorescence and real-time PCR showed that 5 µM Oridonin not only significantly reduced the mRNA expression (Fig. 5c), but also down-regulated the protein level of Mincle in inflammatory macrophage (Figs. 5a, b, d). Moreover, Oridonin significantly down-regulated the protein level of iNOS and phosphorylated-AKT and NF-κB in LPS treated RAW264.7 cells.

Overexpression of Mincle Eliminated the Inhibitory Effect of Oridonin on Inflammation in LPS-Stimulated RAW264.7 Cell

In order to determine whether Mincle is the major target of Oridonin to inhibit inflammation in macrophage, we transfected the RAW264.7 cells with Mincle overexpression plasmid to up-regulate the expression of Mincle. According to the results of real-time PCR and Western blot, it was shown that the mRNA and protein levels of Mincle in RAW264.7 cells were increased after transfection (Figs. 6a, b). The plasmid was then transfected to the Oridonin-treated inflammatory cells, and the results showed that the expression of Mincle was also increased in the cells of this group (Figs. 6c, d). Vector was transfected in cells as the negative control. The results of recovery experiments demonstrated that overexpression of Mincle in Oridonin treated inflammatory macrophage up-regulated the activation of AKT and NF-κB by increasing phosphorylation level of these two proteins (Figs. 6d–g), which suggested that Oridonin inhibits inflammation of macrophage may through down-regulating Mincle and its downstream AKT and NF-κB signaling. Moreover, the real-time PCR and ELISA results showed that overexpression of Mincle restored the expression and secretion of cytokins in Oridonin treated inflammatory macrophage (Figs. 6h–l). The above results indicated that Oridonin inhibits inflammation in macrophage by regulating Mincle/AKT/NF-κB signaling.

DISCUSSION

AKI is a clinical syndrome caused by a variety of etiologies and pathological mechanisms. Renal ischemia–reperfusion injury is a common pathophysiological process in clinic, and it’s one of the main causes of acute kidney injury. Inflammatory response is a complex network involved in renal parenchymal cells and resident immune cells, in which, macrophage plays an important role in kidney injury. Due to changes in the immune microenvironment, macrophages will be polarized into several phenotypes, mainly divided into M1 and M2 types. iNOS expression is a sign of M1 type. In vivo and in vitro studies both found that iNOS expression is increased in the model group, and the expression decreases after treatment. Macrophage-induced C-type lectin (Mincle) is a C-type lectin receptor expressed on activated macrophages and is genetically localized on mouse 6F2 and human chromosome 12P31. Some studies have reported that Mincle participates in the initiation of innate immune response by recognizing pathogen-associated molecular pattern recognition receptors. Some experimental studies have found that after cerebral ischemic injury in mice, the increased infiltration of white blood cells in the injured area can lead to the loss of neurons in the vascular perfusion area, and knockout of Mincle can significantly
slow the ischemic injury caused by middle cerebral artery occlusion. The latest research reveals that Mincle can sense the death of renal tubular epithelial cells that through combine with β-glucosylceramide, accelerating the inflammatory response and delaying elimination of inflammation. \(\text{18)}\) Studies on kidney disease is the same as our research results which have shown that Mincle is highly expressed in both ischemia–reperfusion and cisplatin-induced AKI models, and it is specifically expressed on M1-type macrophages. In summary, the targeted Mincle study may be a new treatment for related diseases caused by inflammatory macrophages. Our study found that the expression of Mincle is increased in AKI mice model constructed by ischemia–reperfusion. According to the results of immunofluorescence co-localization, Mincle is mainly expressed on the kidney macrophages of the model group mice and consistented with the results of Lan’s research. Mincle is induced specifically on M1 macrophages, where Mincle-syk signaling promotes and maintains inflammatory phenotypes of M1 macrophages in acute renal inflammation. \(\text{6)}\) Mincle belongs to C-type lectin receptor (CLR) which could recognize fungi and other forms of microorganisms or aseptic hazards. They induce inflammation through the linker protein CARD9, followed by the assembly of the CARD9-Becl10 complex and typical NF-κB regulation. There is a regulatory relationship

![Fig. 4. Oridonin Suppressed the Expression and Secretion of Inflammatory Factors in LPS-Stimulated RAW264.7 Cells](image_url)
between NF-κB and AKT protein. In the inflammation study of LPS-stimulated macrophages, it is verified that AKT and NF-κB expression is increased. Subsequently, we transfected the high expression plasmid of Mincle into cell which treated with drug intervention, and results found the signal pathway of AKT and NF-κB signaling pathway was activated again. It can be concluded that Mincle can affect the protein expression of this pathway. However, it is not clear how Mincle up-regulates these two signaling pathways, and it is not clear whether the protein is activated through the syk pathway as the reported research says.

Oridonin is a natural diterpenoid compound extracted from Oroxy, and it has a wide range of pharmacological effects, including anti-tumor, anti-inflammatory, and neuroprotection. In the research on the neuroprotective function of Oridonin, Wen’s team found that Oridonin can block insulin resistance by inhibiting autophagy to improve cognitive dysfunction. Lin’s team found that Oridonin can protect against Parkinson’s disease and seizures by reducing neurooxidative stress and other neuroprotective effects. The most thoroughly studied is the antitumor effect of Oridonin. A research team made the drug into inhalable particles to treat non-small cell lung cancer model mice, which showed strong angiogenesis inhibitory effect and pro-apoptotic effect, and played a significant anti-cancer effect. Oridonin can also exert an anti-inflammatory effect. It can inhibit the Notch pathway, induce the polarization of macrophages to switch to an anti-inflammatory phenotype, and suppress the onset of autoimmune neuritis.
It can also alleviate the onset of osteoarthritis by inhibiting the secretion of inflammatory factors and the production of reductive oxidase. Some studies have shown that Oridonin also has significant effects on various kidney diseases. Studies have reported that Oridonin can cooperate with 5-fluorouracil (5-FU) to exert antitumor effects and enhance 5-FU’s cytotoxicity in renal cancer cells. According to the in vivo experiment results of this study, Oridonin can significantly reduce the serum creatinine urea nitrogen in mice with ischemia–reperfusion injury, and significantly reduce its pathological damage. The results also showed that Oridonin can inhibit the inflammatory activity related to suppressing AKT and NF-κB pathway, which is similar to the research results of Oridonin intervention in diabetic nephropathy, and can simultaneously
reduce the infiltration of inflammatory cytokines and the activity of the toll-like receptor 4 (TLR4)/NF-κB signaling pathway. The above results suggested that Oridonin can inhibit Mincle and suppress AKT and NF-κB pathway activity. In vitro experiments, these pathway indicators were increased in LPS-stimulated macrophage inflammation model, and also decreased by Oridonin. The results of restoring experiments showed that, overexpression of Mincle in Oriodonin treated inflammatory macrophage increased the activities of AKT and NF-κB signaling, resulting in up-regulating of expression and secretion of inflammatory cytokins in Oriodonin treated cells, which suggested Oriodonin inhibits inflammation in macrophage may through suppressing Mincle and AKT/NF-κB signaling.

There are many ways to construct AKI animal models, including bilateral ischemia–reperfusion, unilateral IRI with contralateral nephrectomy, cisplatin, diphtheria toxin, LPS, aristolochic acid or folic acid. However, injection of cisplatin can not completely simulate the clinical patients. The model induced by injection of diphtheria toxin tends to prolong the progression of kidney disease, which can mainly lead to renal fibrosis and glomerular sclerosis, as well as mild tubular damage. Aristolochic acid nephropathy is a tubulointerstitial nephritis, and folic acid is also a nephrotoxicity inducer of renal fibrosis, but the clinical incidence of these two is low. IRI-induced acute kidney injury is a global public health concern associated with high morbidity, mortality, and health-care costs. Therefore, we use ischemia–reperfusion to construct an acute kidney injury mice model. Several studies have reported different times of clamping the renal artery. A research team clamped bilateral renal arteries for 35 min before reperfusion, and the changes in serum creatinine were stable after surgery for three days. Therefore, we chose to clamp the bilateral renal arteries for 35 min to prepare a reperfusion model in Wistar rats. In addition, a study was conducted using C57BL/6j mice to clamp the bilateral renal pedicles of Sprague-Dawley (SD) rats for 1h. Based on the previously experimental results, it is found that the reperfusion was resumed after the bilateral renal artery was clamped for 20 min, and the serum creatinine returned to the normal baseline level three days later. Therefore, we chose to clamp the bilateral renal arteries for 35 min before reperfusion, and the changes in serum creatinine were stable after surgery for three days. During the operation, we can observe that the color of the kidney changes from pink to light red, which is caused by renal artery vascular ischemia. After removing the clip 35 min later, we can observe that the color of the kidney returns to normal, the IRI-AKI model was constructed.

In summary, this finding demonstrated that Oriodonin has a significant anti-inflammatory effect, and can protect kidney in AKI from injury, which may mainly involve in inhibiting Mincle and the activity of its downstream signaling of NF-κB and AKT. This study provides a new potential mechanism of Oriodonin treatment for AKI, which can provide options for clinical treatment of AKI.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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