Oridonin Alleviates IRI-Induced Kidney Injury by Inhibiting Inflammatory Response of Macrophages via AKT-Related Pathways

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Background: Acute kidney injury (AKI) is one of the most common complications in clinic, but there is still no effective treatment. Oridonin, extracted from Rabdosia rubescens, has been identified to promote inhibitory effects on tumor, inflammatory and fibrosis by previous study. This study aimed to assess the kidney-protective role of Oridonin in AKI and the underlying mechanism by which Oridonin improves AKI in vivo and inhibits inflammation in LPS-induced bone marrow-derived macrophages (BMDM) in vitro.

Material/Methods: SPF C57BL/6J male mice (8–10 weeks old, body weight 20–25 g) were divided into 3 groups – sham group, AKI group, and Oridonin-treated AKI group – with 6 mice in each group. In the in vitro study, LPS-induced inflammatory BMDM cells were treated with Oridonin and agonist of AKT. The expression and secretion levels of inflammation-related indicators and AKT-related signaling molecules were detected by real-time PCR, ELISA, Western blot, and immunofluorescence. Also, various methods are used to assess renal function and pathological changes.

Results: The results showed that Oridonin treatment significantly improved the serum creatinine and BUN levels in AKI mice. Interestingly, treatment with Oridonin also resulted in decreased the infiltration of macrophages in renal tissues of AKI mice, which was associated with decreased expression and activation of AKT and its related signaling pathways, such as NF-κB and STAT3, suggesting that Oridonin attenuates AKI kidney injury via a mechanism associated with reducing the inflammatory response of macrophages in the AKI kidney. This was investigated in vitro in macrophages, and the results showed that Oridonin reduced the LPS-stimulated inflammatory response in macrophages. Mechanistically, the addition of Oridonin reversed LPS-induced down-regulation of AKT, NF-κB, and STAT3 expression and inflammatory response in macrophages, suggesting that Oridonin has a protective role, via the AKT-related signaling pathways, in reducing the inflammatory response of macrophages in AKI mice. This was further confirmed by adding agonist of AKT of IGF-1 to block the inhibitory effect of Oridonin on inflammatory response in vitro.

Conclusions: Oridonin ameliorates AKI kidney injuries by suppressing AKT-mediated inflammatory response of macrophages.

MeSH keywords: Acute Kidney Injury • Inflammation • Macrophages • Proto-Oncogene Proteins c-akt • Reperfusion Injury

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Background

Acute kidney injury (AKI) is a serious clinical syndrome caused by sepsis, shock, ischemia-reperfusion, and poisoning, characterized with a rapid decline in renal function [1–4]. Nowadays, it is a global public health hazard which harmfully affects patients’ health and has a prevalence of more than 60% during hospitalization [5]. Although the renal replacement therapy has been considered as the most effective treatment for patients with AKI, the mortality rate of severe AKI is still in the range of 50–60% [6]. At present, the intervention treatment for AKI mainly focuses on anti-inflammatory and antioxidation, but still lacks effective treatment measures [7,8].

There are increasing evidences showed that macrophages play an important role in the development of renal inflammation and can mediate inflammation through surface-expressed receptors [4,9]. Recently, it has been reported that vasoactive peptide urotensin II acts on macrophages RAW264.7 and finds that macrophages are activated by AKT signaling pathway to promote inflammation deterioration [10]. Therefore, the AKT signaling pathway may play an important role in renal inflammation response of AKI.

Oridonin, a diterpenoid natural product commonly used in East Asian herbal medicine, is garnering increased attention in the biomedical community due to its extensive biological activities that including anti-tumor, anti-inflammatory, antimicrobial, hepatic fibrosis prevention, and neurological effects [11–14]. Previous studies showed that Oridonin can reduce proteinuria and can mediate kidney damage [15]. In vitro study also demonstrated that it can reduce the secretion of inflammatory cytokines and limit the inflammatory response of LPS-stimulated RAW264.7 cells [16]. Moreover, recent articles using selenium nanoparticles combined with Oridonin to target esophageal cancer cells, demonstrated that Oridonin can induce apoptosis by inhibiting PI3K/AKT and Ras/Raf/MEK/ERK pathways [17]. However, there is currently no report that Oridonin can improve AKI kidney damage and whether it is related to AKT signaling pathways.

In this study, we aimed to explore the effects and mechanism of Oridonin inhibits macrophage activation and protects kidney damage. It reveals that the protective effect of Oridonin on AKI is related to AKT signaling pathway, which provides a new solution for AKI treatment.

Material and Methods

Animals

SPF C57BL/6j male mice (8–10 weeks old, body weight 20–25 g) were divided into 3 groups, sham-operated control group, AKI group and Oridonin treatment group, with 6 mouses in each group. On the day of surgery, we anesthetized the mice (pentobarbital sodium, 50 mg/kg body weight). Mice in the AKI group were reperused after 30 min of bilateral renal artery ischemia. Body temperature was maintained at 37°C throughout all surgical procedures by using a heating device. The mice were euthanized under anesthesia on the third day. Based on the results of our pre-experiment, we injected Oridonin (15 mg/kg/day) intraperitoneally daily from the day of modeling to the Oridonin treatment group and euthanized the mice on the third day of anesthesia. The sham-operated control group was intraperitoneally injected with saline daily as a control. All mouses were housed in the Animal Experimental Center of Southwestern Medical University, providing 12 hours of light and dark circulation, constant temperature and humidity. All of these are in line with the requirements of the Animal Ethics Committee. All the experimental and animal handing procedures were approved by the Committee for the Ethics of Animal Experiments of Southwest Medical University (Permit number: 201812–55).

Chemicals and reagents

Oridonin (purity >98%) was purchased from Biopurify (Chengdu, China). The antibodies of p-NF-κB, p-AKT, p-STAT3, p-iKBα were purchased from Cell Signaling Technology (Beverly, MA, USA). Total RNA extraction kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. Reverse Transcription Kit, Real-time PCR Kit Purchased from Shanghai Promega Bioproducts Co., Ltd. IL-1β, IL-6 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LPS (Escherichia coli 055:B5) was provided from Sigma Chemical Co. (St. Louis, MO, USA).

Serum creatinine and BUN detection

Using the appropriate kit (Nanjing, China), the Bio-Tek microplate reader can measure serum creatinine and urea nitrogen at different wavelengths and calculate the values using the corresponding formula. Values are expressed as mmol/L of serum.

Evaluation of kidney histology

The collected kidney tissue was immediately fixed with 4% paraformaldehyde. Then, the fixed kidney tissue was dehydrated by a fractionated series of ethanol, transparent with xylene, embedded in paraffin, and sliced at a thickness of 4 μm. The tissues were stained with hematoxylin and eosin (H&E).
dyes, and the results of HE staining were observed under a microscope. The periodate was treated for 10 minutes, then Schiff dye solution was added dropwise for 15 minutes, and finally hematoxylin was counterstained. After the dewatering and transparent sealing treatment, the PAS drawing process can be performed.

ELISA

The cell culture supernatant and AKI mouse serum were collected and subjected to corresponding centrifugation. Make the corresponding standard curve according to the kit. The biotinylated antibody and the enzyme-linked reaction substrate were separately incubated, and the corresponding developer and stop solution were added. The absorbance at 450 nm was read using a microplate reader (Bio-Tek). The concentrations of IL-1β, IL-6 and TNFα were calculated from the standard curve and expressed as pg/mg protein based on an appropriate standard curve.

Cell isolation and culture

Primary cell extraction method: exclude normal mouse fat and leg muscle tissue, blunt dissection, respectively, take the corresponding tibia. Rinse with PBS and then disinfect with 70% ethanol. The bones were crushed with the corresponding grinding bowl, the bone marrow was allowed to flow out, and all the cells were obtained after centrifugation. The red blood cells were disrupted, and all the white blood cells were obtained after centrifugation, and induced to differentiate by the supernatant of L929, and the immature cells differentiated into mature BMDM cells. The cells were grown in DMEM low-glucose medium containing 30% L929 supernatant, 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin (GIBCO, USA) and 100 U/ml streptomycin (GIBCO, USA). The cells were incubated in a 37°C incubator containing a constant 5% CO₂.

CCK-8 assay

1×10⁴ cell/well BMDMs were plated in 96-well plates. Incubate at 37°C in a humidified atmosphere containing 5% CO₂. After 24 hours, different concentrations of Oridonin were added. After a further 24 hours, the Cell Counting Kit-8 (CCK-8, MCE, USA) reagent was added according to the manufacturer’s instructions. After incubating for 4 hours in the incubator, the absorbance at 450 nm was read with a microplate reader.

Real-time PCR

Total RNA was extracted from sample using Tiangen Biochemical Technology (Beijing, China) RNA extraction kit. RNA samples were digested with RNase-free DNase I (Tiangen, China). Then, the integrity of the purified RNA was evaluated by the ratio of OD260/OD280. 1 mg total RNA was amplified in cDNA synthesis reactions by using Shanghai Promega Bioproducts (Shanghai, China). The reaction solution was mixed and incubated at 37°C for 15 min, followed by heat inactivation at 95°C for 5 min. The final cDNA reaction mixtures (10 ml) were diluted with 40 ml of water and stored at 20°C until use. Then perform real-time PCR, fluorescence quantitative PCR was carried out at 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, and 40 cycles of reaction. Relative levels of mRNA expression were normalized to GAPDH expression for each gene. The sequence of each gene primer is shown in Table 1.

**Immunofluorescence (IF)**

Cells were fixed in 4% paraformaldehyde for 20 minutes followed by treatment with 0.5% Triton X-100 for 10 minutes. The cells were blocked with 5% BSA for 1 hour, followed by incubation with antibodies against IL-1, IL-6 and TNF-α (Santa Cruz, 1: 200), overnight at 4 degrees. After washing with PBS, the corresponding fluorescently labeled secondary antibody was incubated at room temperature. After dyeing the nucleus, you can observe and photograph under the fluorescence microscope machine (Nikon).

**Table 1. Specific primers for Real-time PCR.**

| Gene | Primer sequence (5’ to 3’) |
|------|---------------------------|
| IL-1β | S: TGCCACCTTTGACAGTGATG  A: AAGGTCCAGGGAAGACAA  |
| IL-6  | S: AAGATGTTGCAATGGCAATTCT  A: AAGTTCATCATCTGTTCTCATCA  |
| TNF-α | S: CATCTTCTCAAAATTCGAGTGGCA  A: TTGGAGTAGAAAGTCACCAACC  |
| MCP-1 | S: CTTCTGGGCTGTGTCCA  A: CGAGCTACTATCGGTAGTCA  |
| iNOS  | S: GTCAGCCACCAACAACTACAGA  A: GTGGACGGGTGCATGTCAC  |
| F4/80 | S: TGGAAAGTGAGTGCGGATAGTG  A: TTCACTGTCTGCTCAACAG  |
| GAPDH | S: AGGTGGTGTGACGGATTG  A: GGGGTCCTGGATGGCAACA  |

**Statistical analysis**

All values are expressed as means±SEM. The comparison between the 3 groups was performed by analysis of variance and Shapiro-Wilk test. The data were not paired and compared with Student’s t-test. The test level was α=0.05, P<0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 7.0.
Results

Oridonin relieved renal injury in classical IRI mouse model

The AKI model was established by clamping the bilateral renal arteries for 30 minutes and then reperfusion. The treatment group was treated with Oridonin. Male C57BL/6J mice in the Oridonin treatment group received intraperitoneal injection for 3 days, and the other groups received intraperitoneal injection of normal saline as a control. The mice were euthanized under anesthesia on the third day. It was observed that serum creatinine and urea nitrogen were significantly increased in the ischemia-reperfusion group compared with the normal control group. Oridonin treatment resulted in a significant reduction in serum creatinine and urea nitrogen compared with the AKI model group. (Figure 1A, 1B). In addition, the treatment of Oridonin significantly reduced the degree of tubular damage and the kidney morphology was restored. (Figure 1C). At the same time, H&E and PAS staining confirmed that the treatment of Oridonin greatly improved the damage of kidney tissue (Figure 1D). Subsequently, it was found by immunofluorescence that the expression of macrophage marker F4/80 was increased in the model group and decreased after Oridonin treatment, which suggested that the macrophage infiltration was significantly reduced after drug treatment (Figure 1E). In summary, Oridonin can improve renal pathological damage and macrophage infiltration of AKI.

Oridonin treatment inhibits inflammation in AKI kidney

Inflammation is the main characteristic in AKI. We examined the secretion and expression of inflammatory factors in AKI kidney cortex and peripheral blood. It was found that consistent with in vitro experiments, Oridonin treatment showed significant inhibition of mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, iNOS and F4/80 expression (Figure 2A). At the same time, after treatment with Oridonin, it can significantly inhibit the secretion of inflammatory factors IL-1β and IL-6 in peripheral blood (Figure 2B). According to the immunohistochemistry results, we found that the treatment of Oridonin can further reduce the protein level of IL-1β, TNF-α and MCP-1 in AKI kidney (Figure 2C). The Western blot results also illustrate the anti-inflammatory effect (Figure 2D). From the above all, we can conclude that Oridonin inhibited the secretion and expression of inflammatory factors in AKI kidney and further reduced inflammation.

Efficacy and safety dose of Oridonin in LPS-stimulated BMDM cells

The chemical structure of Oridonin is shown in Figure 3A. In order to confirm the optimal dose, the cytotoxicity of different concentrations of Oridonin on BMDM cells was determined by CCK8. Firstly, Cell survival of BMDM cells incubated with a range of concentrations of Oridonin (from 2.5 μM to 20 μM) was examined, and the results indicated that Oridonin reduced cell survival in a dose-dependent manner. It was found that when the concentration was higher than 5 μM, cell growth was significantly inhibited (Figure 3B). Thus, we used 5 μM as an optimal dose. Figure 3C shows the cytotoxic effect of AKT agonist IGF1 on BMDM by CCK8, and we found that 75 μg/mL is the optimal dose for action. When LPS was applied to cells, the cell morphology was significantly changed, and it showed increased branching and irregular morphology, developing in a bad direction. Cell morphology was restored after treatment with Oridonin (Figure 3D). From the above results, it showed that Oridonin can partially restore the LPS-induced cell morphology change.

Oridonin inhibits LPS-induced inflammatory response in BMDMs

In order to study the anti-inflammatory effect of Oridonin on cells, 100 ng/mL of LPS was used to establish inflammatory cell model in BMDM cells. The results of real-time PCR showed that expressions of inflammatory factors were significantly increased after LPS stimulation, and, inversely, Oridonin effectively inhibited LPS-induced upregulation of inflammatory factors mRNA level (Figure 4A). At the same time, by detecting the secretion of cytokines in the cell supernatant, we found that Oridonin can obviously reduce the secretion of cytokines IL-1β, IL-6 and TNF-α (Figure 4B). Immunofluorescence results also demonstrated that Oridonin decreased LPS-induced IL-1β, IL-6 and TNF-α protein levels (Figure 4C). From the above results, it suggested that Oridonin obviously reduced the LPS-induced expression and secretion of inflammatory cytokines in BMDM.

Oridonin can significantly inhibit the AKT-related signaling pathway in AKI kidneys

The AKT/NF-κB-related signaling pathway can cause a corresponding inflammatory response. Inflammatory cytokines can further activate the STAT3 signaling pathway. Therefore, the potential effect of Oridonin on the AKT-related signaling pathway was further studied. The results showed that Oridonin treatment inhibited the phosphorylation of AKT, p65, STAT3, and IκBα in AKI kidneys (Figure 5A). We also confirmed by immunohistochemical staining that Oridonin can significantly suppress the upregulation of AKT phosphorylation in AKI kidneys (Figure 5B). Our results suggest that Oridonin protects the kidneys by inhibition of the AKT/NF-κB signaling pathway.

AKT agonist IGF1 upregulated the Oridonin-suppressed inflammation in BMDMs

Consistent with in vivo experiments, our in vitro experiments also showed that Oridonin can effectively inhibit phosphorylation of AKT-associated signaling pathways (Figure 6A).
Figure 1. Oridonin relieved renal injury in a classical IRI mouse model. (A, B) Changes in serum creatinine and urea nitrogen after treatment with Oridonin; (C) The renal tubular injury index; (D) Histological change (H&E and PAS staining) in kidneys in each group of mice; (E) Immunofluorescence results of macrophage marker F4/80. * P<0.05; ** P<0.01; *** P<0.001.
Figure 2. Oridonin treatment inhibits inflammation in AKI kidney. (A) The effect of Oridonin on mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, iNOS, and F4/80, as shown by real-time PCR; (B) Influence of Oridonin on secretion of IL-1β and IL-6 in AKI mice, as indicated by ELISA; (C) Effect of Oridonin on IL-1β, TNF-α, and MCP-1 in tissues as shown by immunohistochemistry; (D) Effect of Oridonin on IL-1β, TNF-α, and iNOS protein levels, as shown by Western blot. * P<0.05; ** P<0.01; *** P<0.001.
To determine the key roles of the AKT signaling pathway in the anti-inflammatory effects of Oridonin, we used the AKT agonist IGF1 to activate AKT in BMDM cells. We found that the addition of the agonist IGF1 in the LPS stimulation group significantly enhanced the expression of p-AKT and attenuated the therapeutic effect of the drug, showing that AKT plays a role in the LPS-induced inflammatory cell model (Figure 6B). Surprisingly, the expression of inflammatory factors IL-1β, IL-6, and TNF-α increased after the addition of IGF1 in the Oridonin treatment group, as determined by PCR and ELISA (Figure 6C, 6D). These results indicate that the anti-inflammatory effect of Oridonin is related to the inhibition of AKT-related signaling pathways.

Discussion

Despite extensive research in the past, there is still a lack of effective drugs for the treatment of AKI. Recently, more and more research has focused on anti-inflammatory and antioxidative treatments with monomer drugs [18]. Oridonin is a diterpenoid compound extracted from Rabdosia rubescens, which has anti-tumor, anti-inflammatory, anti-bacterial, anti-oxidant, and anti-fibrosis functions. In this study, we demonstrated that Oridonin can effectively alleviate kidney damage in IRI-AKI mice. After treatment with Oridonin, the levels of serum creatinine and urea nitrogen in AKI mouse serum were significantly reduced, and renal function was improved. At the same time, Oridonin can effectively reduce the secretion and expression of inflammatory cytokines IL-1β, IL-6, and TNF-α in the AKI model. The in vitro experiments also suggested that Oridonin can significantly reduce the secretion and expression of IL-1β, IL-6, and TNF-α inflammatory cytokines in the LPS-stimulated inflammation BMDM cell model. Further studies found that p-AKT and the related pathway were activated and increased in AKI mice, which were decreased after treatment with Oridonin. Importantly, after treatment with AKT agonist of IGF1, p-AKT was significantly elevated, and the Oridonin-reduced inflammation was also increased in BMDM, suggesting that the mechanism of treatment of AKI by Oridonin is related to inhibition of AKT signaling pathways.

Acute kidney injury (AKI) is defined as a reduction of renal function within a few hours. It is not only a pathological condition of single-organ failure, but also a syndrome manifested by multiple-organ disorders. The incidence of AKI is increasing year by year, accounting for 8–16% of the admission rate [19]. Generally, the common causes of AKI are ischemia-reperfusion injury of various organs, surgery, and organ transplantation. A number of experimental studies have shown that kidney damage is accompanied by infiltration of inflammatory cells, which play an important role in the progression of AKI and produce a pro-inflammatory microenvironment, enhance tissue damage, and promote scar formation [4]. Oridonin has
anti-tumor and anti-inflammatory effects, and it can act on a variety of cells, including immune cells, liver cells, and vascular endothelial cells [16]. Oridonin can also reduce the secretion of inflammatory cytokines and limit the inflammatory response of LPS-stimulated RAW264.7 cells [20]. Many studies have shown that Oridonin can protect the kidneys from injury. Previous studies have shown that Oridonin can prevent damage in diabetic rats and mesangial cells by inhibiting TLR4/p38-MAPK and TLR4/NF-κB signaling pathways [21]. It also reduced proteinuria in a mouse model of spontaneous lupus erythematosus to reduce kidney damage [15]. However, the role of Oridonin in the AKI kidney inflammation model has not been reported in the literature. This study used Oridonin to treat AKI animal models and found that it obviously and significantly improved renal function.

AKT is a serine/threonine (Ser/Thr) kinase that mediates a variety of biological effects and promotes cell survival by phosphorylating a series of apoptosis-regulating proteins. The most important downstream factor of AKT is PI3K [22]. AKT is also an important signaling pathway of apoptosis, which is involved in the regulation of cell survival and anti-apoptosis in various cell types. AKT is also closely related to inflammatory response. AKT can further enhance transcription and translation in the nucleus by activating P65, and secreted inflammatory cytokines can further program the STAT3 signaling pathway and induce

**Figure 4.** Oridonin inhibits LPS-induced inflammatory response in BMDMs. (A) Oridonin inhibits LPS-induced mRNA expression of IL-1β, IL-6, and TNF-α. (B) Oridonin inhibits LPS-induced secretion of IL-1β, IL-6, and TNF-α. (C) Detection of changes in LPS-induced secretion of corresponding inflammatory cytokines by immunofluorescence, magnification: ×200. * P<0.05; ** P<0.01; *** P<0.001.
macrophage polarization [23,24]. Recently, it has been reported that p-AKT is significantly elevated in STZ-induced diabetic rats, and the expression is decreased after berberine treatment [25]. There is also a literature on the use of Withaferin A in the UUO-induced CKD mouse model, showing that Withaferin A can significantly reduce the degree of kidney damage and weaken the expression of p-AKT [26]. Recently, selenium nanoparticles combined with Oridonin have been used to treat esophageal cancer cells, and it has been found to inhibit the expression of p-AKT. IGF1 is a family of insulin-like growth factors, which is mainly secreted and synthesized by hepatocytes and binds to the IGF1 receptor to produce biological effects and plays an important role in regulating growth and development of the body. IGF1 activates the PI3K/AKT signaling pathway and the MAPK signaling pathway [27]. IGF-1 also inhibits apoptosis in neurons and cardiomyocytes by activating the PI3K/AKT signaling pathway [28,29]. Therefore, in the present study IGF1 was used as an activator of the AKT signaling pathway, showing that p-AKT was obviously increased after addition of IGF1 in the Oridonin treatment group, and the corresponding...

Figure 5. Oridonin can significantly inhibit the AKT-related signaling pathway in AKI kidney. (A) The effect of Oridonin on the phosphorylation levels of AKT, P65, IKBα, and STAT3, as indicated by Western blot, and its related statistics analysis chart (right); (B) Detection of the influence of Oridonin on p-AKT protein level by immunohistochemistry. * P<0.05; ** P<0.01; *** P<0.001.
Figure 6. AKT agonist IGF1 upregulated the Oridonin-suppressed inflammation in BMDMs. (A) The effect of Oridonin on phosphorylated AKT, P65, and STAT3 protein levels in LPS-stimulated cells by Western blotting; (B) Western blot analysis showing the effect of adding IGF1 on p-AKT; (C) Changes in IL-1β, IL-6, and TNF-α mRNA levels after IGF1 in the Oridonin treatment group; (D) Effects of IL-1β, IL-6, and TNF-α protein levels secreted by IGF1 in the Oridonin treatment group.

* P<0.05; ** P<0.01; *** P<0.001.
inflammatory response was increased. Therefore, this study is based on previous studies and found that Oridonin treated IRI-AKI was related to inhibition of AKT signaling pathway.

Conclusions

We demonstrated that Oridonin can attenuate IRI-induced AKI kidney damage, presumably through inhibition of macrophage inflammatory response by suppressing AKT-related signaling pathways. Our findings provide a new option for AKI treatment and are expected to provide a new direction for AKI treatment in the future.

Conflict of interest

None.