Summary. A previous study suggested that pyruvate kinase M2 (PKM2) plays a vital role of metabolic reprogramming in the regulation of the innate inflammatory response, while PKM2 is a sensitive biomarker for nephrotoxicity. In this study, we investigated the role and mechanism of PKM2 in development of LPS-induced acute kidney injury. The AKI model of mice was established using LPS. The serum levels of blood urea nitrogen (Bun), creatinine (Cr), and Cystatin C (CysC) were identified using the enzyme-linked immunosorbent assay (ELISA). Hematoxylin and Eosin staining (H&E) was employed to assess pathological changes in kidney tissues of LPS-induced AKI model. Immunohistochemical staining and Western blot analysis were carried out to determine the expression of apoptosis-related factors at protein levels. We found that Bun, CysC, and Cr were significantly increased in the LPS group compared with the control group. The histopathological assay showed model swollen tubular epithelial cells and the presence of vacuolar degeneration in the LPS-induced AKI. In addition, expression levels of PKM2 significantly increased in the LPS group compared with the control group at both protein and mRNA levels (P<0.01). The inhibition of PKM2 by shikonin notably suppressed the expression of HIF-1α and apoptosis-related factors such as BNIP3, Bax, and Caspase-3, while the inhibition of PKM2 by shikonin significantly improved the histopathological symptoms of LPS-induced AKI. This study demonstrated the potential role of PKM2 in LPS-induced AKI and identified PKM2 as a promising therapeutic target in the treatment of AKI.

Key words: Acute kidney injury, LPS, PKM2, Apoptosis, Molecular mechanism

Introduction

Acute kidney injury (AKI) is a common complication in critical illness, and sepsis and septic shock are the most important causes of AKI in critically ill patients with septic associated AKI (SA-AKI) (Zarbock et al., 2014). AKI is associated with high mortality, and accounted for 5% of all hospitalized patients and 30% of critically ill patients (Lameire et al., 2005; Zarbock et al., 2014). Although the incidence of AKI has markedly declined in developed countries, the incidence of AKI is still as high as 15% to 20% in developing countries and the mortality remains over 30% in the majority of studies (Mehrabadi et al., 2016). AKI has been identified as an independent risk factor for death. The patients who survive AKI also face an increased risk of chronic kidney disease (Peerapornratana et al., 2019).

Pyruvate kinase (PK) catalyzes the final step of glycolysis and is composed of four isoenzymes encoded by two distinct genes in mammals. PKs are encoded by the PKLR (1q22) and PKM2 (15q23) genes, with PKLR producing the L (PKL) and R (PKR) isoforms and PKM2 generating the M1 (PKM1) and M2 (PKM2) isoforms (Noguchi et al., 1987; Zhang et al., 2019). In past studies, the comparative proteomics analysis from HK-2 human kidney epithelial cells treated with cisplatin identified that PKM2 and eukaryotic translation elongation factor 1 gamma (EF-1γ) were significantly increased in a kidney cell-specific manner (Kim et al., 2014). Reprogramming of the cell metabolism is also necessary for tumorigenesis and PKM2 plays a decisive role in it (Zahra et al., 2020). In addition, PKM2 is involved in multiple cellular processes including apoptosis, and the up-regulation of PKM2 in Crohn’s disease (CD) might protect intestinal epithelial cells (IEC) against apoptosis, possibly through B cell lymphoma-extra-large (Bcl-xl) to active Caspase-3 and poly (ADP-ribose) polymerase (PARP) (Tang et al., 2015). Increasing evidence indicates the crucial role of metabolic reprogramming in the regulation of the innate inflammatory response. Knockdown of PKM2, hypoxia-
inducible factor 1α (HIF-1α), and glycolysis-related genes decreased lactate production and high mobility group box-1 protein (HMGB1) release, and then targeted aerobic glycolysis in the treatment of sepsis (Yang et al., 2014). Accumulating evidence shows that PKM2 can regulate cell proliferation or apoptosis by affecting cell metabolism or relative gene expression and may act as a specific detection biomarker in the early detection and diagnosis of AKI (Sun et al., 2015; Cheon et al., 2016). However, the role and mechanism of PKM2 in AKI are still rarely reported and remain largely unknown.

Bearing all these in mind, we made a hypothesis that PKM2 may be able to regulate lipopolysaccharide (LPS)-induced AKI through regulating the expression of apoptosis-related factors in the renal tubular epithelial cell. Therefore, we explored the effects and molecular mechanism of PKM2 and its inhibition on LPS-induced AKI and expression of apoptosis-related proteins, which have not been reported.

Materials and methods

Animals

Mice (C57BL/6J) were used in this study. Before the study, all animals were acclimated in an animal room with temperature (22°C), relative humidity (40-50%), and 12 h light-dark cycles for 1 week. The care and management of the animals in this study were approved by Shanghai General Hospital Institutional Animal Care and Use Committee in 2017.

AKI model establishment

A total of 15 mice were randomized into three groups: normal control group, LPS group, LPS + Shikonin group (n=5). LPS (Escherichia coli 055:B5) was provided by Sigma Chemical Co. (St.Louis, MO, USA). The animals in the LPS group were given LPS (5 mg/kg) via intraperitoneal injection every 8 h, 3 times in total and control group received equal amount of PBS; the animals in the LPS + Shikonin group were treated with LPS (5 mg/kg) intraperitoneal injection every 8 h, 3 times in total and shikonin (0.8 μM). 24 h after the LPS treatment, the mice were euthanized and the blood and kidney tissue were collected.

Cell culture

Human HK-2 cell line was acquired from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA). All media were supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Histopathology

Kidney tissue was gathered and fixed in PBS containing 10% formalin. Then the fixed tissues were trimmed in an adapted cube and integrated into paraffin. The paraffin sections were sliced and stained with H&E staining. Finally, the sections were examined by microscope (CX22, Olympus, Tokyo, Japan).

Hematology and serum chemistry

The serum levels of blood urea nitrogen (Bun), creatinine (Cr), and CysC were determined using ELISA kits (R&D, USA) according to the manufacturer’s instructions. Each sample was analyzed in triplicate.

Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using Trizol reagent according to the manufacturer’s instructions. Synthesis of cDNA was utilized in reverse transcription by using Reverse Transcription System reagents (Promega Corporation, Fitchburg, WI, USA). The following sequences of primers were used. The primers of PKM2 were as follows in Table 1.

Western blot

Whole-cell protein extracts were drafted and quantified using a BCA Protein Assay kit (HyClone-Pierce, USA). Protein lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and wet-transferred (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 300 mA for 120 min onto PVDF membranes (Millipore, Billerica, MA, USA). The PVDF membrane was stuck using Tris-buffered saline/tween with 5% non-fat milk at room temperature for 2 h or at 4°C overnight and was incubated with primary antibody and subsequently with an appropriate secondary antibody. Protein bands formed were visualized using an ECL Western Blotting Substrate kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

The data are presented as the mean ± SD. Statistical evaluation of the results was analyzed using one-way ANOVA (Dunnett’s t-test) and two-tailed Student’s t-test. Statistical significance was admitted at P<0.05.
Results

Construction and verification of the animal model of AKI

To elucidate the role of PKM2 in AKI, we first constructed the AKI model induced by LPS treatment. The concentration levels of the Bun, CysC, and Cr were examined for verification, which is the renal function biomarker in the course of AKI. As shown, Bun, CysC, and Cr were substantially increased in the LPS group compared with the control group (P<0.05) (Fig. 1A-C). Meanwhile, H&E staining was utilized to evaluate the histopathological changes in renal tissue. The results showed well-arranged renal tubules and normal renal structure in the control group, but swollen tubular epithelial cells and the presence of vacuolar degeneration in the LPS groups (Fig. 1D). Our results indicated that the AKI model was successfully constructed.

The expression of PKM2 significantly increases in LPS-induced AKI model

Levels of PKM2 in renal tissues were determined to evaluate the PKM2 function in the process of AKI. qRT-PCR and Western blot (WB) were performed to identify the expression of PKM2 at both mRNA and protein levels. According to the results (Fig. 2A,B), the expression levels of PKM2 significantly increased in the LPS group compared with the control group at both mRNA and protein levels (P<0.01). In addition, immunohistochemical staining was also carried out to examine the expression of PKM2, and PKM2 in the LPS group showed higher levels than PKM2 in the control group (Fig. 2C). Our results suggested that the expression of PKM2 markedly increased in AKI model induced by LPS compared with the control group.

The effects of PKM2 on the LPS-induced apoptosis of renal tubular epithelial cells

Next, to explore the role of PKM2 in the LPS-induced apoptosis of renal tubular epithelial cells, HK-2 cells were treated with different concentrations of LPS (0, 0.1, 1, 10, 100 μg/ml). We detected the expression of PKM2 at protein levels. The results showed that the expression of PKM2 was sharply increased in the LPS group compared with the control group (P<0.01), and the...
expression of PKM2 was dose-dependent on the concentration of LPS (Fig. 3). In addition, the expression levels of Cleaved-Caspase-3, Cleaved-Caspase-9, HIF-1α, and BNIP3 in each group of cells were also detected by WB to verify whether LPS induced apoptosis of renal tubular epithelial cells, and affected the expression level of PKM2 and the downstream signaling pathways. Compared with the control group, Cleaved-Caspase-3, Cleaved-Caspase-9, HIF-1α, and BNIP3 concentrations increased considerably with the LPS concentration (Fig. 3). The results indicated that the expression of PKM2 was significantly increased in the AKI model, while apoptosis-related factors showed higher expression in the AKI model induced by LPS in comparison to normal control group.

Inhibition of PKM2 downregulates the expression of apoptosis-related factors in HK-2 cells

According to the previous results and considering the excessive destruction of renal tissue by high LPS concentration, a concentration of LPS (0.1 μg/ml) was selected to treat HK-2 cells. As an inhibitor of PKM2, shikonin was utilized to inhibit the expression of PKM2 in HK-2 cells. WB was used to determine the expression of PKM2 and apoptosis-related proteins, and the results showed that shikonin at 0.8 μM significantly inhibited the expression of PKM2, HIF-1α and apoptosis-related factors (such as Cleaved-Caspase-3, Cleaved-Caspase-9 and Bax) and recovered the expression of anti-apoptosis proteins such as Bcl-2 in HK-2 cells (Fig. 4). Our results
suggested that downregulation of PKM2 by shikonin can suppress cell apoptosis by regulating the expression of apoptosis-related factors.

**Inhibition of PKM2 alleviates LPS-induced AKI by regulating apoptosis in vivo**

To verify the role and mechanism of PKM2 in vivo, after successful AKI model construction by LPS, the expression of PKM2 in mice was inhibited by shikonin. The results of H&E staining showed that partial renal hemorrhage was caused in the LPS group. Kidney injury caused by LPS was significantly inhibited in the group treated with shikonin as expected (Fig. 5A). The levels of serum Bun, Cr and CysC were measured by ELISA to evaluate renal function in mice. It was visible by the results that the serum Bun, Cr, CysC levels in the LPS+shikonin group were discernibly lower than the LPS group (Fig. 5B-D), indicating the alleviation of AKI by shikonin treatment. This is in agreement with the previous H&E staining results, which revealed that shikonin effectively inhibited kidney damage induced by LPS. Subsequently, IHC and WB were employed to detect the expression of the PKM2 and apoptosis-related proteins, in which the expression of HIF-1α, PKM2, Caspase-3, Cleaved-Caspase-3, Caspase-9, cleaved-Caspase-9, BNIP3, and Bax were significantly inhibited by shikonin at the molecular level compared to the LPS group (Fig. 5E-F). Compared to the LPS group, Bcl-2 was up-regulated in the shikonin group at protein levels (Fig. 5F). Our results indicated that PKM2 constituted an essential role in AKI and inhibition of PKM2 by shikonin significantly improved the pathological symptoms of AKI in mice.

**Discussion**

Acute kidney injury (AKI) is a serious nephrotic syndrome with high incidence in hospitalized and critically ill patients. Worldwide, AKI is a critical disease with poor clinical prognosis and lack of effective treatment, with high morbidity and mortality and many complications. Clinically, renal ischemia-reperfusion, nephrotoxic drugs and sepsis are the most common predisposing factors of AKI. At present, there are no drugs that can effectively improve the kidney injury and enhance the repair after kidney injury in AKI patients, so the mortality of severe AKI patients has been high in recent decades (Negi et al., 2018; Moore et al., 2018; Mercado et al., 2019; Perazella, 2019). The pathogenesis of AKI is very complicated and is currently unclear. Nephrotoxicity, ischemia and hypoxia, inflammatory...
response imbalance, glomerular hemodynamic changes, autophagy, apoptosis, oxidative stress, etc., are all related to the occurrence and development of AKI (Guo et al., 2019). Therefore, it is important to improve the clinical outcome and quality of life of patients with AKI and to fully understand the molecular mechanism of the occurrence and development of AKI and develop new diagnosis and treatment methods.

Lipopolysaccharide, also known as endotoxin, is an important part of the outer membrane of gram-negative bacteria. A large number of studies have shown that LPS can induce inflammation, sepsis and acute kidney injury (Aki et al., 2017; Pfalzgraff and Weindl, 2019; Ciesielska et al., 2021). In this study, we used lipopolysaccharide (LPS) to induce the AKI model of mice. The serum Bun, CysC, and Cr were used as the criteria to assess the AKI model (Simsek et al., 2012). At 24 h after treating by LPS, the presence of vacuolar degeneration was observed by histopathological assay, and the serum Bun, CysC, and Cr increased markedly compared to the control group. The AKI model was successfully established.

PKM2 is expressed in many normal cells or cancer cells. Accumulating evidence shows that PKM2 with

![Fig. 4. Shikonin treatment regulates the LPS-induced expression changes of PKM2 and apoptosis-related factors. WB was used to examine the expression of PKM2 and apoptosis-related factors in HK-2 cells treated by LPS (0.1 μg/ml) or LPS + shikonin (0.8 μM).]
proliferative capacity or anabolic function is important for cancer cell proliferation, and regulation of PKM2 enzymatic activity can influence cell proliferation (Israelson et al., 2013; Lunt et al., 2015). Pyruvate kinase catalyzed the decisive step in glycolysis, transferring a phosphate group from phosphoenolpyruvate (PEP) to ADP to produce pyruvate and ATP (Hosios et al., 2015). As a biomarker of AKI, PKM2 was used for the early detection of AKI (Cheon et al., 2016). In this study, we found that the expression of PKM2 significantly increased in renal tissues treated by LPS compared to the normal control group. Furthermore, shikonin, a commonly used PKM2 inhibitor (Chen et al., 2011; Li et al., 2014), was used in vitro and in vivo for suppressing the expression of PKM2, exploring the regulation of AKI and apoptosis-related proteins by PKM2. It was demonstrated that the AKI-induced upregulation of HIF-1α, and apoptosis-related factors were significantly attenuated by shikonin. Activation of HIF-1 usually occurs in human cancers either as result of hypoxia or genetic alteration, and HIF-1 activates transcription of genes encoding proteins that are involved in key aspects of metabolism, cell survival, invasion, and metastasis (Semenza, 2010). HIF-1 was classified as an O2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (Wang et al., 1995). PKM2 can interact directly with the HIF-1α subunit and promote transactivation of HIF-1 target genes (Luo et al., 2011).

Fig. 5. Shikonin treatment attenuates the LPS-induced AKI. A. H&E staining was used to evaluate histopathological changes of AKI model; ELISA kit was used to detect the serum Bun (B), CysC (C), and Cr (D). E. Immunohistochemical staining was used to examine the protein expression of PKM2 and HIF-1α in kidney tissues collected from mice in corresponding groups. F. WB assay was used to examine the protein expression of apoptosis-related proteins in kidney tissues collected from mice in corresponding groups. *P<0.05, **P<0.01, ***P<0.001 vs. control group. +P<0.05, ++P<0.01, LPS group vs LPS+shikonin group.
This study lacked the research on the regulatory mechanism between HIF-1α and PKM2 in LPS-induced AKI, which will be our follow-up work. In conclusion, this study demonstrated the molecular mechanism of PKM2 in cell apoptosis on LPS-induced AKI, and recognized that the inhibition of PKM2 may be an effective strategy in the treatment of AKI.

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