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Targeting Oct2 and P53: Formononetin prevents cisplatin-induced acute kidney injury

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ABSTRACT

Nephrotoxicity is one of major side effects of cisplatin in chemotherapy. Therefore, there is an urgent medical need to develop drugs that may protect kidney from toxicity. In previous study, we found that it showed the protective effects of formononetin against apoptosis by upregulating Nrf2. In this study, we investigated the renoprotective effect of formononetin against cisplatin-induced AKI and tried to elucidate the possible mechanisms. The amelioration of renal function, histopathological changes, and apoptosis in tubular cells was observed after formononetin treatment. Formononetin decreased expression of organic cation transporter 2 (Oct2) and increased the expressions of multidrug resistance-associated proteins (Mrps), which might result in a decrease accumulation of cisplatin in tubular cells after AKI. 5-Bromo-2-deoxyuridine (BrdU) and Ki-67 staining assay indicated that formononetin could promote the renal tubular cells proliferation after cisplatin nephrotoxicity. Moreover, formononetin regulated cyclins and pro-apoptotic proteins to involve the regulation of cell cycle. Furthermore, formononetin decreased p53 expression via promoting the overexpression of murine double minute 2 (MDM2) and MDMX. Taken together, formononetin provided protective effects by promoting proliferation of surviving renal tubular cells and inhibiting apoptosis after cisplatin-induced AKI.

1. Introduction

Since the accidental discovery in late 1960s, cisplatin has been widely used for chemotherapy to treat a variety of cancers (Siddik, 2003; Wang and Lippard, 2005). Cisplatin and related platinum-based therapeutics for the treatment of certain cancer types are remarkably high. However, cisplatin is also well known for its side effects, particularly, nephrotoxicity which limits the effects and efficacy in therapy (Yao et al., 2007). It is manifested clinically as lower glomerular filtration rate, involving necrosis as well as apoptosis of renal tubular cells. The causative mechanisms of cisplatin nephrotoxicity include renal inflammation, oxidative stress, vasoconstriction of the renal microvasculature, apoptosis and/or necrosis of tubular epithelial cells (Miura et al., 1987; Safirstein et al., 1987; Pabla and Dong, 2008). In this setting, it may lead to the loss of renal tubular cells and the denudement of the basement membrane (Bonventre and Yang, 2011). It is reported that the protection against renal injury might be associated with transporters (Liu et al., 2012; Guo et al., 2013). Cisplatin was taken up by Oct2 and excreted by Mrp2 in kidney. On the other hand, Mrp2 and Mrp4 are apical transporters on the brush-border membrane that efflux chemicals into urine. We hope that the accumulation of cisplatin in renal tubular cells would be reduced via decreasing level of Oct2 and promoting level of Mrp2.

Kidneys have the great capacity for regeneration. The mechanism may be considered about rapidly triggering extensive cellular proliferation after tubular damage caused by AKI. The major processes of regeneration are linked to the proliferation of surviving cells and replacement of necrotic tubular cells with functional cells (Sharfuddin and Molitoris, 2011). The injury models are known to result in death of substantial epithelial cell followed by a proliferative repair response (Nony and Schnellmann, 2003a). This repair process has been clearly observed in several animal models subjected to acute injury.

P53, the well-known tumor suppressor protein, could promote processes of AKI in cisplatin-nephrotoxicity by inhibiting proliferation of

Abbreviations: AKI, acute kidney injury; MDM2, murine double minute 2; Oct2, organic cation transporter 2; Mrp2, multidrug resistance-associated protein 2; BrdU, 5-bromo-2-deoxyuridine.

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proximal tubular cells. It is reported that pifithrin-α, a pharmacological inhibitor of p53, could partially inhibit apoptosis induced by cisplatin in proximal tubular cells (Zhou et al., 2010). The recent study showed that the activation of p53 in renal tubular cells partially localized with apoptosis in nephrotoxicity. Importantly, tubular cell apoptosis, renal tissue damage, and renal failure were ameliorated in p53-deficient mice. The same phenomenon was observed in wild-type animals treated by pifithrin-α (Wei et al., 2007).

Considering about the side effects of cisplatin and the importance of the p53 signaling pathway in apoptosis and proliferation, we hypothesized that the apoptosis and proliferation of proximal tubular cells after renal injury might be related with p53 signaling.

Formononetin is the main compound of red clover plants, which belongs to herbal isoflavone. Besides, we have found that formononetin could regulate the anti- and pro-apoptotic proteins to protect the renal injury (Di Huang et al., 2016). It is reported that formononetin could show markedly hair regrowth by regulating p53 (Kim et al., 2016). However, the effect of formononetin on renal regeneration has never been reported. In the present study, we aimed to determine the effects of formononetin on promoting renal regeneration in cisplatin-induced toxicity, and further to explore the potential mechanisms *in vivo* and *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

Formononetin (purity > 98%), metformin and cisplatin were purchased from Dalian Meilun Medical Science and Technology Company Ltd (Dalian, China). P53 activator AB143228 was purchased from Abcam Company. P53 inhibitor pifithrin-α was obtained from Selleck Chemicals LLC.

2.2. Animals

Male Wistar rats (220–250 g) in this study were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number: SCXK 2008-0002). The rats were housed with a 12:12 h light-dark cycle with food and water available. The rats were randomly allocated into five groups (Control: saline i.v.; AKI: cisplatin i.p. at the dose of 12 mg/kg; Formononetin o.p. at the dose of 15 mg/kg, 50 mg/kg, 75 mg/kg with cisplatin treatment). Formononetin (15, 50 or 75 mg/kg) or vehicle (10% HPBCD in 500 mM phosphate pH 7.0) alone was treated to rats by oral gavage twice daily for 5 days. On the third day, 4 h after formononetin or vehicle treatment, rats were injected intraperitoneally physiological saline or cisplatin at a dose of 12 mg/kg. On the forth and 5th day, 4 h after formononetin or vehicle treatment, animals were euthanized under anesthesia (pentobarbital sodium, 65 mg/kg, intraperitoneal injection). The blood and kidney were collected at 48 h after cisplatin treatment.

At the end of the experiment, the rats were killed. Blood was collected and processed to obtain the plasma. Kidney and liver tissues were collected for the various measurements.

All animals used in the study were performed in accordance with the National Institutes of Health guidelines for the care and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China.

2.3. Cell culture

HK-2 cells, a proximal tubular cell line derived from human kidney, were purchased from KeyGEN BioTECH (Nanjing, China) and expended in DMEM/F12 (Gibco BRL, Carlsbad, CA), supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA), 100 U/ml penicillin (Sigma, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37 °C in a humidified atmosphere composed of 5% CO_2.

2.4. Biochemical assay

The levels of blood urea nitrogen (BUN), creatinine were measured by detection kits based on the manufacturer's instructions obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China).

2.5. Kidney histology

After being killed, part of the kidney tissue of each rat was instantly fixed in 10% formalin for at least 24 h, embedded in paraffin, sectioned at 5 μm. Paraffin-embedded slides from kidney were stained with hematoxylin & eosin (H&E). The changes of tubular damage were graded as follows: 0-trace, normal or mild tubular cell swelling, and/or vacuolar degeneration; (I) areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving <25% of cortical tubules; (II) similar changes involving >25% but <50% of cortical tubules; (III) similar changes involving >50% but <75% of cortical tubules; (IV) similar changes involving >75% of cortical tubules. For 5-bromo-2-deoxyuridine (BrdU) staining, rats were injected intraperitoneally with the BrdU solution (50 mg/kg body weight, twice per day for 5 days) following the experimental treatment (Miyaji et al., 2001). Kidney sections were prepared and stained using a BrdU staining kit (Roche, Indianapolis, IN). Antibody against Ki-67 was purchased from Proteintech Group (USA). The number of positively stained cells was counted in at least 5 randomly selected fields for each tissue section.

2.6. Renal slice preparation and uptake study

Rats' kidney cortical tissues were cut into slices as previously described (Zhu et al., 2012). Slices (300 μm in thickness, surface area approximately 0.15 cm^2) of rat kidney were stored on ice immediately after kidney removal.

Slices were pre-incubated in 6-well culture plates for 3 min with oxygenated buffer at 37 °C, and then were transferred to 24-well culture plates containing 1 ml fresh oxygenated buffer (120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) containing metformin (100 μM) for further incubation. Metformin uptake was terminated by removing the buffer and washing the slices with ice-cold saline at 15 and 30 min. After homogenization, the accumulated concentrations of metformin in kidney slices were determined by LC-MS/MS.

2.7. Cell cycle analysis

To identify the proliferation of HK-2 cells, 5×10^5/well HK-2 cells were seeded in 6-well plates and treated with cisplatin (20 μM) or both cisplatin and formononetin (10 μM) for 24 h. Cells were harvested and fixed at least 1 h with 70% ice-cold alcohol at 4 °C. The proliferative cells were detected by FxCycle™ PI/RNAse Kit according to the manufacturer's instructions and analyzed by flow cytometric analysis (FACSCalibur, BD, USA).

2.8. Caspase-3 activity

The effect of formononetin on caspase-3 activity was analyzed. Caspases-3 activity in kidney was measured as the manufacturer's instructions obtained from KeyGen Biotech (Nanjing, China).

2.9. Quantitative real-time PCR

Total kidney RNA was extracted using RNAiso Plus® Reagent Kit (Takara Biotechnology, Dalian, China) following the manufacturer's instructions and then reverse-transcribed cDNA with PrimeScript® RT