Treatment with catalpol protects against cisplatin-induced renal injury through Nrf2 and NF-κB signaling pathways

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Received November 20, 2018; Accepted May 20, 2020

DOI: 10.3892/etm.2020.9077

Abstract. Cisplatin (CP) is one of the most widely used chemotherapy drugs for cancer treatment, but it often leads to nephrotoxicity. It is well known that catalpol exhibits antioxidant and anti-inflammatory functions, thus the present study aimed to investigate the potential protective effects of catalpol on CP-induced kidney injury in rats, in addition to determining the underlying mechanisms. Sprague-Dawley rats were treated with 25, 50 or 100 mg/kg catalpol for two days, injected with 20 mg/kg cisplatin and catalpol on day 3 and sacrificed on day 4. The histological analysis of isolated kidney tissues was performed using hematoxylin and eosin staining, cleaved caspase-3 expression levels were analyzed using western blotting and the expression levels of inflammatory cytokines in the tissues, including tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, IL-10 and inducible nitric oxide synthase (iNOS) were evaluated using ELISAs. Furthermore, the mRNA and protein expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), kelch-like ECH-associated protein 1 (Keap1), NF-κB and inhibitory κB (IκB) were determined using reverse transcription-quantitative PCR and western blotting, respectively. The results revealed that the treatment with catalpol prevented the histopathological injury and renal dysfunction caused by CP. In addition, catalpol significantly suppressed the CP-induced apoptosis of tubular cells, inhibited the CP-induced upregulation of TNF-α, IL-1β, IL-6, IL-8 and iNOS and promoted the production of the anti-inflammatory cytokine IL-10. Additionally, the mRNA and protein expression levels of Nrf2, HO-1 and IκB in the kidney tissues were increased, whereas the expression levels of Keap1 and NF-κB were significantly decreased following the treatment with catalpol. In conclusion, these results suggested that catalpol may inhibit CP-induced renal injury and suppress the associated inflammatory response through activating the Nrf2 and inhibiting the NF-κB signaling pathways, respectively.

Introduction

Cisplatin (CP) is a chemotherapeutic drug that has been used clinically for decades in patients with malignant tumors; however, nephrotoxicity, which is the major side effect of CP treatment, has greatly limited its application as a treatment (1,2). Thus, there is an urgent requirement to develop a novel therapeutic agent that protects against CP-induced renal injury and obtain novel insights into the treatment of patients with cancer undergoing CP-based chemotherapy regimens (3,4).

The molecular mechanisms underlying CP-induced nephrotoxicity are complex (5). It has been reported that inflammation and apoptosis are associated with the development of nephrotoxicity (6,7); proinflammatory factors stimulated by CP, including tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6 and IL-8 have been observed to serve essential roles in the pathogenesis of CP-induced renal injury (8,9). Thus, the inhibition of the inflammatory response and apoptosis could be a promising therapeutic strategy for attenuating CP-induced nephrotoxicity. NF-κB, an important regulator of cytokine induction, promotes the expression of multiple proinflammatory genes (10). NF-κB has been demonstrated to have a vital role in the progression of CP-induced renal injury (11). Previous studies have found that the severity of CP-induced nephrotoxicity is related to the activation of NF-κB, and conversely, the inflammatory response and severity of nephrotoxicity can be attenuated through the inhibition of NF-κB activity (12,13). In addition, numerous studies have suggested a role for nuclear factor E2-related factor 2 (Nrf2) in the regulation of physiological processes that serve to inhibit the development and progression of CP-induced renal damage (14,15). It has been reported that the absence of Nrf2 exacerbates CP-induced nephrotoxicity, whilst the phar-
macological activation of Nrf2 has been observed to inhibit CP-mediated nephrotoxicity (8). Thus, the pharmacological activation of Nrf2 is considered to be an important molecular target to prevent CP-induced renal damage.

*Rehmannia glutinosa* is a traditional herbal medicine that has been used to enhance the functions of the liver, kidney and heart (16). In certain cases, *Rehmannia glutinosa* has also been used to treat diabetes, anemia and urinary tract infections (17). A previous study extracted the effective component in *Rehmannia glutinosa* and identified it as catalpol, whose molecular formula is C_{36}H_{42}O_{6} (18). Studies have since revealed that catalpol exhibits anti-inflammatory and anti-apoptotic effects (19, 20). In addition, catalpol was found to have important functions in protecting against lipopolysaccharide-induced acute lung injury through suppressing the inflammatory response (21). In other studies, catalpol inhibited apoptosis in hydrogen peroxide-induced cardiac myocytes through the caspase pathway and ameliorated hepatic insulin resistance in type 2 diabetes by acting through the 5'-AMP-activated protein kinase/NADPH oxidase 4/P13K/AKT signaling pathway (22, 23). However, to the best of our knowledge, no previous study has been conducted to examine the inhibitory roles of catalpol in CP-induced renal injury. In the present study, the potential protective effects of catalpol in CP-induced kidney injury were investigated in an in vivo rat model and the underlying molecular mechanisms were subsequently investigated. The results suggested that catalpol may protect against CP-induced apoptosis and inflammation in tubular cells by inhibiting and activating the NF-κB and Nrf2 signaling pathway, respectively.

**Materials and methods**

**Reagents.** Catalpol was purchased from Sigma-Aldrich; Merck KGaA; the Urea Nitrogen Diacytymonoxide Test (Kinetic) kit were obtained from Tiangen Biotech Co., Ltd. TNF-α (cat. no. ZB-10764C-R9648), IL-1β (cat. no. ZB-10119C-R9648), IL-6 (cat. no. ZB-10135C-R9648), IL-8 (cat. no. ZB-11167C-R9648), IL-10 (cat. no. ZB-10119C-R9648) and iNOS (cat. no. ZB-10740C-R9648) ELISA kits were purchased from ZellBio GmbH. TRizol® reagent was obtained from Invitrogen; Thermo Fisher Scientific, Inc. Primary antibodies against cleaved caspase-3 (cat. no. 9661; 1:1,000), Nrf2 (cat. no. 12721; 1:1,000), heme oxygenase-1 (HO-1; cat. no. 86806; 1:1,000), inhibitory kβ (IκB; cat. no. 70641; 1:100), ECH-associated protein 1 (Kaepl; cat. no. 8047; 1:2,000) and NF-κB p65 (cat. no. 8242; 1:2,000) were purchased from Cell Signaling Technology, Inc.

**Animal studies.** A total of 40 male Sprague-Dawley rats (age, 8 weeks; mean body weight, 392±15 g) were purchased from the Animal Center of the Military Medical University (Chongqing, China) and were housed at 2 rats/cage in a light-controlled environment at 24±1°C, 40-80% humidity, with 12-h light/dark cycles and with access to food and water *ad libitum* throughout the experimental period. The experiments were approved by the Animal Care and Use Ethics Committee of the Military Medical University.

**Experimental design.** Rats were randomly divided into five groups (n=8/group): i) CP group, which was subjected to a single injection of 20 mg/kg CP intraperitoneally on day 3; ii) control group, which was administered intraperitoneally with an equal volume of the saline solution (20 ml/kg) instead of CP or catalpol; and iii) CP and catalpol (CP + cat) groups treated with 25, 50 or 100 mg/kg catalpol for 2 days and 20 mg/kg CP and catalpol on day 3. A 2-ml blood sample was collected on day 4 from the retroorbital venous sinus following anesthetization by the intraperitoneal injection of 300 mg/kg 10% chloral hydrate aqueous solution. The blood samples were centrifuged at 1,509 x g for 15 min at 4°C and stored at -80°C until further use. The rats were sacrificed by decapitation following the drawing of blood. The dose of catalpol was chosen based on a previous study (24).

**Renal function analysis.** To examine the renal injury, the expression levels of blood urea nitrogen (BUN) and creatinine in the serum were analyzed using a biochemical AutoAnalyzer (Cobas® 8000; Roche Diagnostics GmbH), according to the manufacturer's protocol.

**Histological analysis using hematoxylin & eosin (H&E) staining.** For histopathological evaluation of the renal injury, kidney tissues were obtained from the rats and were subsequently fixed in 10% formaldehyde at room temperature for 24 h and embedded in paraffin. The 5-µm sections were heated at 60°C for 1 h, before being de waxed in xylene and rehydrated using a descending ethanol series. Hematoxylin and eosin (H&E) staining was then performed on sections, with hematoxylin for 10 min room temperature and eosin for 5 min at room temperature. Stained sections were visualized using a light microscope (magnification, x200) by a pathologist in a blinded manner. Renal histopathological changes including cellular necrosis, loss of brush border, interstitial edema and tubule dilatation were evaluated using the following criteria: i) 0=none; ii) 1=≤25%; iii) 2=25-49%; iv) 3=50-74%; and v) 4=≥75% (6).

**TUNEL assay.** To evaluate the apoptotic ability of the cells in the cortex following CP-induced renal injury, a TUNEL assay was performed using the TUNEL apoptosis detection kit (cat. no. C1098; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Apoptotic cells were observed and counted in five randomly selected fields using a light optical microscope (magnification, x200). Cells with a brown nucleus were positive cells. The number of apoptotic cells in proportion to the total number of cells was used to calculate the apoptotic index.

**Evaluation of inflammatory cytokines.** To determine the expression levels of TNF-α, IL-1β, IL-6, IL-8, IL-10 and iNOS in renal tissues. The renal tissues (100 mg) were homogenized with 1 ml PBS (pH 7.4, 100 mM) and PathScan® Sandwich ELISA lysis buffer (cat. no. 7018; Cell Signaling Technology, Inc.) was used to lyse the tissues before centrifugation at 3,660 x g for 10 min at 4°C. TNF-α (cat. no. ZB-10764C-R9648), IL-1β (cat. no. ZB-10119C-R9648), IL-6 (cat. no. ZB-10135C-R9648), IL-8 (cat. no. ZB-11167C-R9648), IL-10 (cat. no. ZB-10108C-R9648) and iNOS (cat. no. ZB-10108C-R9648) ELISA kits were purchased from ZellBio GmbH. The cytokine expression levels were quantified using a Bio-Rad Benchmark Microplate reader (Bio-Rad Laboratories, Inc.).
and iNOS (cat. no. ZB-10740C-R9648) ELISA kits were used according to the manufacturer's protocol.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The temperature protocol for reverse transcription was as follows: 42˚C for 45 min, 99˚C for 5 min and 5˚C for 5 min. qPCR was performed using the SYBR® Green PCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequences of primers (Shanghai Institute of Biological Sciences) are listed in Table I. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min. 45 cycles at 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 10 sec. Relative mRNA expression levels were calculated using the 2-ΔΔCq method (25) and normalized to the internal reference gene GAPDH.

Western blotting. Renal tissue samples were ground in liquid nitrogen and lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) for 30 min. Total protein was quantified using a bicinchoninic acid assay and 50 µg protein was separated using 12% SDS-PAGE for 90 min. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked in TBS with 5% skimmed milk for 2 h at room temperature. The membranes were incubated with primary antibodies (all from Cell Signaling Technology, Inc.) against GAPDH (cat. no. 5174; 1:1,000), cleaved caspase-3 (cat. no. 9661; 1:1,000), Keap1 (cat. no. 8047; 1:2,000), Nrf2 (cat. no. 12721; 1:1,000), HO-1 (cat. no. 86806; 1:1,000), NF-κBp65 (cat. no. 8242; 1:2,000) and IκB (cat. no. 76041; 1:100) overnight at 4°C. Following the primary antibody incubation, the membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2370; 1:5,000; Santa Cruz Biotechnology Inc.) for 1 h at 37°C. The protein-antibody complexes were visualized using Pierce™ Fast Western Blot Kit, ECL Substrate (cat. no. 35050; Thermo Fisher Scientific, Inc.) with a chemiluminescence instrument (Tanon Science and Technology Co., Ltd.). Protein expression was quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.). Experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS, Inc.) and data are presented as the mean ± SD. Statistical differences between groups were deter-

Table I. Primer sequences for reverse transcription-quantitative PCR.

| Target gene | Forward | Reverse |
|-------------|---------|---------|
| NF-κB       | ACCTGCAGTTGATGCTGAT | CCTGTCAACCAGGCAGTTAT |
| Keap1       | TGCAATGGATCTGCTTCACCTTTTTCAGGAA | TGACCCGAGAATCCTTCCTTTTCCAGTTTC |
| Nrf2        | GCAACTCGAGAAGGAACAGG | GGAATGTCGCTGCCAAAAGC |
| HO-1        | CCCAGAGGTCAGGTTGC | TGCCTTTGCTTCTATTC |
| IκB         | TGCCGATGTTGACAGCCTTC | GACATCAGACACCAAGTCA |
| β-actin     | CCACCTGCCGATCCTCTT | GCATCGGAAACGCTCATT |

Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; IκB, inhibitory κB.
mined using one-way ANOVA with a Tukey's post hoc test for multiple comparisons. Histological scores were compared using Kruskal-Wallis statistical test followed by Dunn's post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

**Catalpol prevents CP-induced renal injury.** To analyze the protective roles of catalpol on CP-induced renal injury, the expression levels of BUN and creatinine in the serum were determined. The expression levels of serum BUN and creatinine in the CP group were significantly increased compared with the control group; however, serum BUN and creatinine expression levels were significantly reduced following the treatment with catalpol in a dose-dependent manner compared with CP treatment alone (Fig. 1). These results suggested that CP may stimulate renal injury, which may be prevented by catalpol treatment.

**Effects of catalpol on CP-induced renal histopathological changes.** To investigate the functions of catalpol on CP-induced renal injury, the histological appearance of kidney tissues was analyzed. Normal morphology was observed in the control group (Fig. 2A); however, tubular epithelial damage, intratubular cast formation and tubular dilatation were detected in CP-treated tissues (Fig. 2B). Catalpol was observed to prevent the CP-induced histological disturbances in renal tissues in a dose-dependent manner compared with CP treatment alone (Fig. 1). These results suggested that CP may stimulate renal injury, which may be prevented by catalpol treatment.

**Catalpol inhibits the apoptosis of tubular cells in the kidney tissues with CP-induced renal injury in vivo.** The apoptosis of tubular cells serves essential pathogenic roles in CP-induced renal injury (6). In the present study, the apoptotic ability of the cells was determined using TUNEL staining and analyzing caspase-3 activity. Low numbers of apoptotic cells were detected in the kidney tissues of the control group (Fig. 3A-F); however, following the treatment with CP, the number of apoptotic cells in the kidney tissues was significantly increased compared with the control group (Fig. 3B-F). Furthermore, catalpol treatment significantly inhibited the number of apoptotic cells in the kidney tissues compared with that in the CP group (Fig. 3C-F). In addition, cleaved caspase-3 expression levels were increased in the CP group compared with the control group, whereas catalpol treatment significantly decreased these levels (Fig. 3G and H). The results demonstrated that the apoptotic rate of cells isolated from the CP group was significantly increased compared with the control group; however, this effect was significantly reversed following the treatment with catalpol, suggesting that the increased cell apoptotic ability and subsequent CP-induced renal injury may be inhibited by catalpol treatment.

**Catalpol inhibits the secretion of cisplatin-induced inflammatory cytokines.** Previous studies have demonstrated that the production of inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8, IL-10 and iNOS, served important roles in CP-induced kidney injury (8,9). To investigate the anti-inflammatory functions of catalpol, the effects of catalpol on the expression levels of CP-induced inflammatory cytokines were determined. ELISAs revealed that the production of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-8 and iNOS in kidney tissues were significantly increased in the CP group compared with the control group, whereas the secretion of the anti-inflammatory cytokine IL-10 was significantly decreased compared with the control group (Fig. 4). The treatment with catalpol significantly inhibited the production of CP-induced TNF-α, IL-1β, IL-6, IL-8 and iNOS, whilst significantly increasing the expression of IL-10 in a dose-dependent manner compared with the CP group (Fig. 4). These results indicated that the CP-induced inflammatory response may be...
suppressed by the treatment with catalpol, which may represent a novel therapeutic candidate for the treatment of CP-induced inflammation and renal injury.

**Effects of catalpol on the expression levels of Nrf2, HO-1, IκB, Keap1 and NF-κB.** The mRNA and protein expression levels of Nrf2, HO-1 and Keap1 were analyzed using RT-qPCR and western blotting, respectively. The mRNA and protein expression levels of Nrf2 and HO-1 were significantly reduced, whilst the expression levels of Keap1 were significantly increased following the treatment with CP compared with the control group (Fig. 5). Meanwhile, catalpol treatment significantly increased the expression levels of Nrf2 and HO-1, whilst significantly decreasing the expression levels of Keap1 compared with the CP group (Fig. 5). The activation of NF-κB serves an important role in the induction of proinflammatory mediators and nuclear translocation of the NF-κB transcription factor is preceded by the degradation
IκB-α (8). To determine whether catalpol affected NF-xB activity, NF-xB p65 and IκB-α expression levels were analyzed using RT-qPCR and western blotting. Catalpol significantly increased IκB-α expression levels and inhibited nuclear NF-xB p65 expression levels following CP-induced renal injury (Fig. 5).

Discussion

CP is one of the most effective chemotherapy drugs; however, its therapeutic application is restricted by nephrotoxicity, the major CP-induced side effect that consequently leads to renal injury (26,27). It has been reported that CP-stimulated injury in the kidney is closely associated with inflammation and apoptosis (28). For example, Mitazaki et al. (29) demonstrated that IL-6 was involved in the regulation of oxidative stress during the development of CP-induced nephrotoxicity; Lee et al. (30) reported that mice with an IL-1α deficiency were more resistant to CP-induced acute renal failure compared with the control group; and Kim et al. (31) reported that the expression levels of IL-10, an anti-inflammatory cytokine, were significantly decreased following treatment with CP. Additionally, in another study, IL-10 was found to protect the kidney against renal ischemia and CP-induced injury (32). In the present study, the serum levels of BUN and creatinine in the CP group were significantly increased, whereas the treatment with catalpol reduced their expression levels in a dose-dependent manner. These results suggested that CP may promote renal injury, which may be subsequently prevented by catalpol treatment; this conclusion could be further validated by analyzing the expression levels of urinary microalbumin and β2-microglobulin in future studies. Moreover, catalpol inhibited the production of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8 and iNOS and subsequently reduced the damage of CP-induced renal dysfunction. Furthermore, the treatment with catalpol resulted in the increased secretion of the anti-inflammatory cytokine, IL-10, which may protect the normal function of kidney. Thus, catalpol may be a promising therapeutic candidate for the treatment of CP-induced kidney injury.

However, to the best of our knowledge, the underlying molecular mechanisms of catalpol-mediated protection on kidney function were unknown, so they were further investigated in the present study. NF-xB is a pleiotropic transcription factor with important functions in the intestinal immune system. NF-xB family members control the transcriptional activities of various promoters of proinflammatory cytokines, cell surface receptors, transcription factors and adhesion molecules involved in intestinal inflammation (10). NF-xB is located in the cytoplasm of most cells as an inactive complex with unprocessed precursor proteins or IκB. The degradation of these precursor proteins enables NF-xB dimers to translocate to the nucleus and induce the expression of specific target genes (13). Notably, the transcription factor has an essential role in the transcriptional regulation of cytokines, such as IL-1β, IL-6 and TNF-α, and the activation of the NF-xB pathway promotes the expression of inflammatory parameters associated with severe renal injury (6). The results of the present study revealed that the activation of NF-xB was significantly inhibited by catalpol, indicating that catalpol may attenuate the CP-induced inflammation of renal injury through suppressing the NF-xB signaling pathway.

In addition, the apoptosis of tubular cells is considered as one of the pathogenic mechanisms that contributes to diseases associated with renal injury (33). Nrf2 exerts cytoprotective and antiapoptotic effects, and is found present as an inactive complex in the cytoplasm with Keap1. In the nucleus, Nrf2 activates the expression of the HO-1 gene, which subsequently alleviates oxidative stress-induced cellular damage (8,14). In addition, HO-1 is a phase II detoxifying enzyme and an anti-inflammatory reactive protein (34). In the present study, catalpol treatment significantly reduced the apoptotic ability of tubular cells in vivo. Furthermore, as an important protein involved in the regulation of antioxidant proteins and the inhibition of cell apoptosis, the expression levels of Nrf2 and associated genes, including Keap1 and HO-1, were evaluated following the treatment with CP and catalpol. The results revealed that CP suppressed the levels of genes involved in the Nrf2 axis, whereas catalpol promoted the expression of the aforementioned genes, which suggested that catalpol may...
inhibit the apoptosis of tubular cells and subsequently protect the kidney against CP-induced renal injury.

In conclusion, the results of the present study demonstrated that catalpol was able to attenuate CP-induced inflammation and apoptosis during renal injury through the activation and inhibition of the Nrf2 and NF-kB signaling pathway, respectively. These findings provided novel insights into the potential protective roles of catalpol against kidney injury, suggesting that catalpol may be a potential therapeutic candidate in the treatment of CP-induced renal damage. However, as a widely used chemotherapy drug, the anti-tumor functions of CP are mainly attributed to its regulatory roles on cell apoptosis, thus, the therapeutic outcome of combined CP and catalpol treatment for patients with solid tumors remains unknown and requires further investigation.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Science Foundation of China (grant no. 81130011) and National Institutes of Health (grant nos. DK064005 and DK091239).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JHZ and JZ conceived and designed the present study. JZ performed the experiments; LL analyzed the data. FL and ZW contributed to interpretation of data. JZ wrote the manuscript and JHZ edited the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by the Animal Care and Use Ethics Committee of the Military Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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