Self-Protection against Triptolide-induced Toxicity in Human Hepatic Cells via Nrf2-ARE-NQO1 Pathway*

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ABSTRACT  **Objective:** To find the signaling pathway of triptolide (TP)-induced liver injury and to reveal whether NF-E2-related factor 2 (Nrf2) plays an important role in cellular self-protection. **Methods:** The L-02 and HepG2 cells were cultured and treated with various concentrations of TP. The cell viability was observed, and the cell medium was collected for detecting the aspartate aminotransferase (ALT), alanine aminotransferase (AST), lactate dehydrogenase (LDH), superoxide dismutase (SOD) and L-glutathione production (GSH) levels. Nrf2 and its downstream target NAD(P)H: quinine oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) expression, the nuclear translocation of Nrf2, and the binding ability of Nrf2 and antioxidant response element (ARE) were also identified. Meanwhile, shRNA was used to silence Nrf2 in L-02 cells to find out whether Nrf2 plays a protective role. **Results:** The viability of the L-02 and HepG2 cells treated with TP decreased in a dose- and time-dependent manner, and TP (20–80 μg/mL) markedly induced the release of ALT, AST and LDH (P<0.05, P<0.01), reduced the levels of SOD and GSH (P<0.01), and increased the intracellular reactive oxygen species. Meanwhile, TP augmented the Nrf2 expression in L-02 and HepG2 cells (P<0.05, P<0.01), induced Nrf2 nuclear translocation, increased the Nrf2 ARE binding activity, and increased HO-1 and NQO1 expressions. Nrf2 knockdown revealed a more severe toxic effect of TP (P<0.05, P<0.01). **Conclusions:** Human hepatic cells treated with TP induced oxidative stress, and led to cytotoxicity. Self-protection against TP-induced toxicity in human hepatic cells might be via Nrf2-ARE-NQO1 transcriptional pathway.

KEYWORDS  triptolide, human hepatic cells, NF-E2-related factor 2, oxidative stress, liver injury

Triptolide (TP), a diterpenoid epoxide, is the major active component of the *Tripterygium wilfordii* Hook f., which has been widely used in Chinese medicine to treat autoimmune diseases, such as rheumatoid arthritis and lupus erythematosus.¹ It has been reported to have activities against mouse models of cancer, renal fibrosis and polycystic kidney disease.² It has exhibited many pharmacological activities, including immune modulation, anti-inflammatory, proapoptotic and antiangiogenesis activities. Thus, TP has also risen to wide acceptance in the medical research in the West.²⁻⁴ Several putative target proteins of TP have been reported, including polycystin-2, ADAM10, DCTPP1, TAB1, and XPB. However, the toxicity of TP limits its therapeutic potential. It has a narrow margin between the therapeutic and toxic doses and could cause severe injury to the circulatory, digestive, reproductive and urogenital systems.⁵⁻⁶

Liver is one of the major sites of TP-induced toxicities. It is reported that acute liver injury is one of the main causes of death in mice after TP administration.³ Although many studies have reported the hepatotoxicity of TP,⁶⁻⁸ the signaling pathway of TP-induced liver injury has not been fully elucidated yet. Until now, no available strategy has been found...
to protect against TP-induced liver injury. A possible mechanism for TP-induced liver injury was related to oxidative damage induced by reactive oxygen species (ROS). In liver, hepatocytes response to oxidative stress are regulated by NF-E2-related factor 2 (Nrf2), a redox-sensitive nuclear transcription factor. Oxidative stress can lead Nrf2 to translocate into the nucleus, where it binds to the antioxidant response element (ARE), a cis-acting element on the promoter of multiple cytoprotective genes. The binding of Nrf2 to ARE results in transactivation of ARE-dependent gene expression. The antioxidant enzymes such as heme oxygenase-1 (HO-1) and NAD(P)H: quinine oxidoreductase 1 (NQO1) are encoded by typical Nrf2-target genes dependent on Nrf2-ARE binding. Nrf2-dependent antioxidant pathway has been regarded as the most important mechanism in the cell for protection against oxidative stress. Li, et al reported that Nrf2 played an important role in protection against TP-induced kidney injury. Thus, we hypothesized that activation of Nrf2-dependent antioxidant pathway could protect against TP-induced hepatotoxicity.

In the present study, TP-induced cell injury and oxidative stress were examined in human hepatic cells. The underlying Nrf2-ARE pathway was also identified to reveal the mechanisms of TP-induced hepatotoxicity.

METHODS

Experimental Cells
Human normal liver L-02 cells and human hepatoma HepG2 cells were purchased from Chinese Center for Type Culture Collection, Wuhan University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂ at 37 °C.

Instruments, Drugs and Regents
The instruments used were as follows: Bio-Rad 680 microplate reader (USA), Olympus IX51 immunofluorescence microscopy (Japan), Becton Dickinson Biosciences FACSCalibur (USA), Bio-Rad electrophoresis apparatus (USA), Bio-Rad VL Chemi-Smart 3000 imaging device (USA).

TP (97% purity, verified by high performance liquid chromatography at Testing and Analysis Center, School of Pharmacy, Nanjing University of Chinese Medicine) was purchased from Zelang Medical Technology Co. (Nanjing, China). TP was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions which were filtrated and preserved at −20 °C. Stock solution was diluted to the desired concentrations before use. DMSO content within the media should not exceed 0.1% (v/v).

DMEM, FBS and DNA-protein binding detection kit were purchased from Gibco (CA, USA), anti-β -actin antibody was purchased from CST (MA, USA), anti-Nrf2 antibody was purchased from RD (MN, USA), anti-HO-1 antibody and anti-NQO1 antibody were purchased from Santa Cruz (CA, USA). Tetramethyl rhodamine isothiocyanate (TRITC)-coupled secondary antibodies was gained from Jackson ImmunoResearch Laboratories (PA, USA). The negative control shRNA and shRNA targeting Nrf2 were obtained from Genechem Technology Co. (Shanghai, China). Lipofectamine™2000 was from Invitrogen (CA, USA). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), superoxide dismutase (SOD) and L-glutathione (GSH) detection kit were obtained from Jiancheng Bioengineering Institute (Nanjing, China).

Cell Viability Assay
The L-02 and HepG2 cells were cultured in 96-well plates (1 × 10⁴ cells/mL) and grown overnight. And then the cells were treated with various concentrations of TP (final concentrations were 0.01, 0.1, 1, 10, 20, 40, 80, 160 and 320 μg/mL in L-02 cells, or 0.00004, 0.0004, 0.004, 0.04, 0.4, and 16 μg/mL in HepG2 cells) or control (0.1% DMSO). After treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for another 4 h. After that, medium was removed and 150 μL DMSO was added to each well in order to dissolution. A value was measured at 490 nm using a microplate reader.

Determination of Hepatic AST, ALT, LDH, SOD and GSH Production
L-02 cells were seeded in 6-well at a density of 5 × 10⁵ cells/mL, grown overnight and treated with the presence or absence of various concentrations of TP (10, 20, 40, and 80 μg/mL) for 24, 36 and 48 h. After treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for another 4 h. After that, medium was removed and 150 μL DMSO was added to each well in order to dissolution. A value was measured at 490 nm using a microplate reader.
**Determination of ROS Production**

The procedures of cell culture and drug administration were the same as described above. The cells were collected and washed with phosphate buffer solution (PBS), and then the cells were mixed with dichlorofluorescin diacetate (DCFH-DA) staining solution (10 μg/mL) at a density of 1 × 10⁶ cells/mL. The suspension was incubated at 37 °C, 5% CO₂ for 20 min, mixed every 3–5 min so that the cells could contact with the probe sufficiently. After that, the samples were washed with DMEM solution for 3 times and analyzed by flow cytometry at the excitation of 488 nm and the emission wavelength at 525 nm.

**Western Blot Analysis of Protein Expression**

The L-02 cells and HepG2 cells were seeded in 6-well at a density of 5 × 10⁵ cells/mL. The procedures of L-02 cell culture and drug administration were the same as described above. While HepG2 cells were treated with TP at various concentrations (4, 40, 400 and 4,000 μg/mL) for 48 h. After treatment, the cells were washed by PBS and lyzed in ice-cold lysis buffer (containing 1% phenylmethanesulfonyl fluoride). The total proteins were quantified by bicinchoninic acid (BCA) assay and the Nrf2 protein expressions in L-02 cells and HepG2 cells, and the HO-1, NQO1 expressions in L-02 cells were detected by Western blot method: the 50 μg proteins were electrophoresed on 10% sodium dodecylsulphate (SDS) polyacrylamide gel (40 mA, 1 h). After electrophoresis, proteins were transferred to PVDF membranes (100 V, 90 min), and the membranes were blocked in 5% non-fat dry milk-PBST buffer (PBS containing 0.1% Tween-20) overnight at 4 °C. Blots were washed 3 times followed by incubation in the 1:5000 dilutions of secondary antibodies (anti-Nrf2, 1:200, 100 μL/piece) overnight at 4 °C. After 3 washes in PBS, the climbing pieces were incubated with TRITC-coupled secondary antibodies at 37 °C in the darkness and nuclei were counterstained with 4', 6-diamidino-2-phenylindole. The fluorescence signals were imaged by the immunofluorescence microscopy.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assay (EMSA) for determining the Nrf2 and ARE binding activities in the L-02 cells was performed using a DNA-protein binding detection kit. In brief, the ARE oligonucleotide probes were synthesized and annealed with TE buffer at 90 °C, then gradually cooled to 4 °C, 10 μg of nuclear extracts and 0.5 μL of labeled oligonucleotide were incubated at room temperature for 20 min along with binding buffer. The DNA protein complexes were separated by 6% non-denaturating polyacrylamide gel electrophoresis (PAGE) at 100 V and then transferred to the NC membrane at 100 V for 45 min. The complexes were visualized by chemiluminesence imager and the luminescence intensity was quantitated by VL Chemi-Smart 3000 imaging device.

**Transient Transfection with Nrf2- shRNA**

Negative control RNAi (5′-TTCTCCGAA C G T G T C A C G T - 3′), NFE2L2-RNAi (5′-GGCATTTCACTAAACACAAC-3′) and the expression vectors (hU6-MCS-UBiquitin-EGFP-IRES-puromycin) were used in the experiment. The L-02 cells were transiently transfected at the 80% confluence: 1.6 μg of Nrf2-shRNA (or negative control shRNA) and 4 μL of lipofectamine were separately diluted in 100 μL of Opti-MEM medium, two kinds of mixtures were mingled and incubated for 20 min. The samples were then added to the 12 well-plates (200 μL/well), incubated at 37 °C, 5% CO₂ for 36 h. Western blot analysis was used to determine the level of Nrf2 expression. After treatment with 20 μg/mL of TP, the cell morphologies were observed and levels of AST, ALT, SOD and GSH were measured.

**Statistical Analysis**

Data were shown as mean ± standard deviation (X ± s). Western blot analyses were performed by quantitative software Image Lab 3.0 (Bio-Rad, USA). SAS software was used for statistical analysis. ANOVA and t-test were applied for comparison of the means of two or multiple groups. A value of P<0.05 was considered significant.
RESULTS

TP Induces Toxicity in L-02 and HepG2 Cells

The viability of L-02 cells treated with TP decreased in a dose- and time-dependent manner (Figure 1A). TP at the concentrations of 10–80 μg/mL was selected as cytotoxic concentrations for subsequent assessments. Meanwhile, the viability of HepG2 also decreased in a dose- and time-dependent manner (Figure 1B), and 4–4000 μg/mL exposure were selected as cytotoxic concentrations of TP. As shown in Figures 1C–1E, 36 h incubation with various concentrations of TP (from 20–80 μg/mL) markedly induced the release of ALT, AST and LDH (P<0.05, P<0.01).

TP Induces Oxidative Stress in L-02 Cells

The intracellular ROS was increased by 8.7%, 15.5%, 21.8% and 30.3% at 10, 20, 40 and 80 μg/mL of TP, respectively (Figure 2A). TP reduced the intracellular levels of SOD and GSH in a dose-dependent manner, with 80 μg/mL of TP resulting in the most severe depletion (P<0.01, Figures 2B and 2C).

TP Induces Nrf2 Accumulation and Nuclear Translocation

Western blot analysis depicted that TP...
augmented the expression of Nrf2 in L-02 and HepG2 cells in a dose-dependent manner ($P<$0.05, $P<$0.01, Figures 3A and 3B). In agreement with this notion, the results of immunofluorescence detection of Nrf2 in L-02 cells confirmed that TP increased the Nrf2 expression, and revealed that TP induced Nrf2 nuclear translocation (Figure 3C). The Nrf2-ARE binding activity increased after TP treatment in L-02 cells, and reached peak values at concentration of 20 $\mu$g/mL, and declined thereafter (Figure 3D).

**TP Affects the Expressions of HO-1 and NQO1**

HO-1 and NQO1 expressions increased in cells treated with TP compared with control ($P<$0.05). Interestingly, the increase of HO-1 expression was in a dose-dependent manner, while NQO1 expression reached the peak at a dose of 20 $\mu$g/mL, and declined thereafter (Figure 4).

**Nrf2 Gene Knockdown Enhanced Cytotoxicity Induced by TP**

Nrf2-shRNA transfection led to a knockdown of Nrf2 protein as determined by Western blot analysis ($P<$0.01, Figure 5A). Morphological analysis revealed a more severe toxic effect of TP in Nrf2 knockdown cells compared with that of control cells (Figure 5B). Nrf2 knockdown significantly increased the release of ALT and AST of L-02 cells after treated with TP for 36 h ($P<$0.05, $P<$0.01, Figures 5C and 5D).

Intracellular GSH and SOD decreased after TP treatment ($P<$0.01), and were at lower levels in Nrf2 knockdown cells compared with that of control cells treated with the same concentration of TP ($P<$0.05,
that pathways related to chemical-induced oxidative stress may involve in TP-induced hepatotoxicity.

Nrf2 plays an important role in oxidative damage induced by various toxic xenobiotics.\(^1\) In this study, TP-induced cell injury and oxidative stress in human hepatic cells were enhanced when Nrf2 was knocked down by shRNA, which revealed the protective role of Nrf2 against TP-induced hepatotoxicity. It has been reported that the effect of one typical Nrf2 activator (sulforaphane, SFN) can protect against TP-induced liver toxicity in mice.\(^1\) Thus, the authors suggested
that SFN could be a promising agent in protecting livers from TP-induced damages. This study confirmed the important role of Nrf2 in TP-induced hepatotoxicity. Nrf2 is activated by changes in the redox state of cells and functions to restore homeostasis by upregulating antioxidant, xenobiotic-metabolizing, and other cytoprotective enzymes. We found that TP affects the transcriptional activation of Nrf2-ARE-NQO1, a well-known antioxidant mechanism. NQO1 is considered as a detoxification enzyme due to its ability to reduce reactive and toxic hydroquinones. The two-electron reduction of quinones bypasses semiquinone production and prevents the generation of ROS. It is reported that Nrf2 plays a major role in cellular protection against oxidative stress through the ARE-mediated induction of NQO1. Interestingly, we found that Nrf2-ARE binding activity and NQO1 expression reached peak values at TP concentration of 20 μg/mL, and declined with higher concentrations of TP treatment. These results suggested that cellular self-protection via Nrf2-ARE-NQO1 transcriptional pathway was obviously at low or moderate concentrations of TP treatment. While high concentration of TP induced severe toxicity, and suppressed the effects of cellular self-protection. In this study, HO-1 expression was in a TP dose-dependent manner and was independent of Nrf2 ARE binding activity. Thus HO-1 might be involved in other Nrf2-dependent antioxidant pathway, which requires clarification in future studies. Yeligar, et al also reported that ethanol regulates HO-1 and NQO1 transcription by different signaling pathways.

In summary, human hepatic cells treated with TP induced oxidative stress and led to cytotoxicity. As a defensive response against TP-induced oxidative stress and toxicity, Nrf2 was activated, which binding with ARE, regulated the expression of NQO1. This work elucidates a possible signaling pathway of TP-induced liver injury and provides new insights into the development of strategies to prevent or alleviate TP-induced hepatotoxicity.

Conflicts of Interest
There are no conflicts of interest for any of the authors.

Author Contribution
Zhou LL and Zhou XP conceived and designed the experiments; Zhou LL, Zhou C, Liang XW, Feng Z and Liu ZP performed the experiments; Zhou LL, Zhou C and Wang HL analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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