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Knockout of hepatic P450 reductase aggravates triptolide-induced toxicity

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ABSTRACT

Triptolide, the primary active component of Tripterygium wilfordii Hook F, has various pharmacological activities but also a narrow therapeutic window. Cytochrome P450s are proposed to be responsible for the hydroxylation of triptolide *in vitro* and CYP3A induction by dexamethasone can increase the metabolism of triptolide and decrease the hepatotoxicity in rat. However, triptolide-induced toxicity has not been investigated in an animal model having a suppression of P450 activities. Here we compared the toxicological effects and toxicokinetics of triptolide between liver-specific cytochrome P450 reductase (CPR) knockout (KO) mice (abolished hepatic P450 activities) and wild-type (WT) control mice after a single oral gavage of triptolide at 0.5 mg/kg or 1.0 mg/kg. A low toxic dose of triptolide at 0.5 mg/kg for WT mice resulted in severe toxicities including death in KO mice. Changes in serum biochemistry, hematology and histopathology further indicated much more severe toxicities in multiple organs in KO mice compared to WT mice after triptolide administration. The mono-hydroxylated metabolites of triptolide detected in the blood of WT mice were undetectable in KO mice, accompanied by much higher triptolide levels in the blood and tissues including the liver, kidney, and spleen determined by LC–MS/MS. Taken together, our results confirmed that inactivation of hepatic P450s abolishes the ability in metabolism of triptolide in the liver, subsequently resulting in an increase in bioavailability and toxicity of triptolide *in vivo*. It is suggested that P450 inhibition/inactivation might pose a significant health risk in the clinic use of triptolide.

1. Introduction

Extracts of the Tripterygium wilfordii Hook. f. (TWHF) have been used in China for centuries to treat autoimmune and inflammatory diseases (Lin et al., 2007). Triptolide (triptolide), a diterpenoid triepoxide purified from TWHF, has been identified as a key biologically active component of TWHF and used as a unique quality control of Tabellae Glucosidorum Tripterygii Totorum (Leigongtengduodai Pian) in pharmaceuticals (Qiu and Kao, 2003). However, the clinical uses of triptolide are limited by its narrow therapeutic window and high toxicity (Ni et al., 2008), shown as a high incidence of side effects mainly associated with damages to the digestive, urogenital, circulatory, and reproductive systems, as well as bone marrow (Zhang et al., 2009).

Drug-induced toxicity can be caused either by parent compounds or by their reactive metabolites that are generated through biotransformation primarily in the liver (Srivastava et al., 2010). The cytochrome P450 superfamily members contribute to approximately 80% of this process (Evans and Relling, 1999). Pretreatment with dexamethasone, a CYP3A inducer significantly increased one of the mono-hydroxylated metabolites of triptolide and decreased its hepatotoxicity in rat (Ye et al., 2010). In addition, *in vitro* data indicated that both CYP3A4 and CYP2C19 were involved in the metabolism of triptolide into mono-hydroxylated metabolites (Li et al., 2008). However, among the three major mechanisms for P450 involvement in drug–drug interactions, i.e. induction, inhibition, and possibly stimulation, inhibition appears to be the most important in terms of known clinical problems (Guengerich, 1997). Therefore, studying the effects of P450 inhibition or inactivation on triptolide-induced toxicity, which remains unclear so far, could provide important information for safe use of triptolide in the clinic.

Recently, a mouse model of liver-specific cytochrome P450 reductase (CPR) knockout (KO) has been developed (Gu et al., 2003; Henderson et al., 2003), in which the CPR gene encoding the sole

Abbreviations: ALB, albumin; ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; Cre, creatine; CPR, cytochrome P450 reductase; DMSO, dimethyl sulfoxide; Glc, glucose; Hb, hemoglobin; KO, knockout; PLT, total number of platelets; RBC, the number of red blood cells; TC, total cholesterol; TG, triglycerides; TP, total protein; TWHF, Tripterygium wilfordii Hook f; WBC, total number of white blood cells; WT, wild-type.

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electron donor for all microsomal P450s is deleted specifically in the liver, resulting in about 95% loss of the hepatic microsomal activity. It has been demonstrated that hepatic P450-dependent metabolic activation of acetaminophen and detoxification of aristolochic acid are inhibited in the CPR KO mouse (Gu et al., 2005; Xiao et al., 2008). Thus, the liver CPR KO mouse will be an appropriate model to evaluate the effects of hepatic P450 dependent metabolism on drug-induced toxicity *in vivo*.

The present work compared the toxicological effects and blood/tissue levels of triptolide and its metabolites between CPR KO mice and WT littermates. Our results demonstrate that inactivation of hepatic P450s abolishes the hydroxylation of triptolide in the liver, subsequently leading to an increase in the blood/tissue levels of triptolide and its toxicity *in vivo*, which suggests a critical role of hepatic P450s in the metabolic detoxification of triptolide.

2. Materials and methods

2.1. Chemicals and animal treatments

Triptolide (99% purity) was purified from the Chinese traditional herb Tripterygium wilfordii Hook. f. (TWHF) by Prof. Yuanchao Li (Shanghai Institute of Materia Medica, Shanghai, China). The CPR KO mice were a gift from Prof. Xinxin Ding (Wadsworth Center, Albany, NY, USA). Procedures for animal breeding and genotyping were reported previously (Xiao et al., 2008). Eight to twelve weeks old KO mice and their WT littermates in a mixed C57BL/6 and 129/Sv genetic background were used in this study. Animal use protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica, China. All animals were provided with a certified standard diet and tap water *ad libitum* during the experiments.

As the LD50 of triptolide was reported to be 0.86 mg/kg (i.p.) in mice (Zhou et al., 2005), more clinically relevant doses were used in our animal experiments. In survival experiments, mice ($n = 8$) were administered a single dose of triptolide at 0.5 mg/kg in saline with 2.5% dimethyl sulfoxide (DMSO) by oral gavage, and the numbers of surviving animals were recorded within 7 days after the treatment. For toxicity/toxicokinetics experiments, mice ($n = 5$/group for each time-point) were treated with a single dose of triptolide at either 0.5 mg/kg or 1.0 mg/kg by oral gavage. The control groups received vehicle only.

2.2. Toxicological study

Mice were euthanized within 24 h after triptolide treatment. After drawing blood samples from orbital venous sinus, the liver, kidney, spleen, thymus, heart and duodenum were collected promptly and fixed in 10% neutral buffered formalin for histological examination as described previously (Xiao et al., 2008). The tissue sections were stained with hematoxylin and eosin (H and E). For semi-quantitative assessment of the extent of tissue toxicity, the severity of lesions in the tissues was graded as +++, severe; ++, moderate; +, mild; or −, negative, as described previously (Gu et al., 2005).

The serum levels of blood urea nitrogen (BUN), creatine (Cre), alanine aminotransferase (ALT), aspartate transaminase (AST), glucose (Glc), triglycerides (TG), total cholesterol (TC), total protein (TP) and albumin (ALB) were determined using an automatic HITACHI Clinical Analyzer Model 7080 (Hitachi High-Technologies Corporation, Tokyo, Japan).

Hematological parameters were obtained on an ADVIA 2120 Hematology System (Bayer HealthCare Diagnostics Division, Tarrytown, NY).

2.3. Toxicokinetics study

Mice were divided into four groups to collect blood and tissues: (1) 0.5 mg/kg triptolide treated WT mice; (2) 0.5 mg/kg triptolide treated KO mice; (3) 1.0 mg/kg triptolide treated WT mice and (4) 1.0 mg/kg triptolide treated KO mice. After triptolide treatment, blood samples were collected from at 5, 15, 20, 30, 60, 90 and 120 min ($n = 5$/time-point for each group) respectively. Plasma was separated by centrifugation at 3000 rpm for 5 min and kept at −80 °C until analysis. Tissues including the liver, kidney and spleen were also collected from individual mice at 15, 30, 60 and 120 min ($n = 5$/time-point for each group) after dosing, weighed and then homogenized in saline (1.0 g wet weight/mL) on ice. Triptolide was then extracted from the plasma or the tissue homogenates including liver, kidney and spleen for each sample with an equal volume of ethyl acetate and then dried under nitrogen. The residues were reconstituted in 50 μL of mobile phase for analysis.

The quantification of triptolide in samples was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with an autosampler coupled to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies Inc., Palo Alto, CA, USA). Separations were conducted under isocratic conditions. The mobile phase consisting of acetonitrile and water (50:50, v/v) was set at a flow rate of 300 μL/min. An electrospray interface in positive ionization mode was used. ESI source parameters were as followed: high purity drying-gas (N_2) flow rate 8 L/min, temperature 350 °C, capillary voltage 3500 V, nebulizer pressure 25 psi. Multiple reaction monitoring (MRM) was used to quantify triptolide (m/z 361 $[M+H]^+ \rightarrow$ 128, fragmentor 110 eV, collision energy 70 eV). Analytical data were processed using the Mass Hunter software package (Agilent Technologies Inc., Palo Alto, CA, USA) consisting of qualitative and quantitative software.

Standard curves for triptolide were prepared by spiking known amounts of triptolide standard into plasma or tissue homogenate samples prepared from untreated mice. Triptolide concentrations in biological samples were determined by comparisons with the standard curves. Pharmacokinetic parameters were calculated using the Kinetica software (version 4.4.1; Thermo Fisher Scientific Inc., Woburn, MA, USA).

2.4. Statistical analysis

All data are expressed as mean ± S.D. and analyzed with Student's *t*-test, one-way analysis of variance followed by Newman–Keuls comparisons, or two-way ANOVA analysis followed by Bonferroni post tests, $p < 0.05$ was considered statistically significant.

3. Results

3.1. KO mice were more sensitive to triptolide-induced toxicity than WT mice

We first observed the survival rate in WT and KO mice within 7 days. After a single dose of triptolide by oral gavage at 0.5 mg/kg, no death was observed in WT mice, whereas all of KO mice were dead in 5 days (Fig. 1).

Furthermore, we compared changes in various toxicological parameters in WT and KO mice at 24 h after dosing of triptolide at 0.5 mg/kg or 1.0 mg/kg. Serum biochemical analysis showed dose-dependent increases in serum levels of ALT and AST in both WT and KO mice after triptolide treatment, but the increments were dramatically greater in KO mice than in WT mice (Fig. 2A and B). The serum levels of BUN, Cre and TG were dose-dependently increased in KO mice after triptolide treatment, whereas no changes were observed in WT mice (Fig. 2C–E). The serum level of Glc was dose-dependently decreased by triptolide in both WT and KO mice, with a significantly lower level in KO mice than in WT mice at 0.5 mg/kg dosage (Fig. 2G). The serum level of TC in untreated KO mice was lower than in WT mice (Fig. 2F). However, after triptolide treatment at 1.0 mg/kg, the serum level of TC in KO mice was similar to that in WT mice (Fig. 2F). The serum levels of TP and ALB were dose-dependently decreased by triptolide in KO mice (Fig. 2H and I), whereas only TP was decreased in WT mice at 1.0 mg/kg dosage.

Hematological analysis showed that the total number of platelets (PLT) was dose-dependently decreased in KO mice after

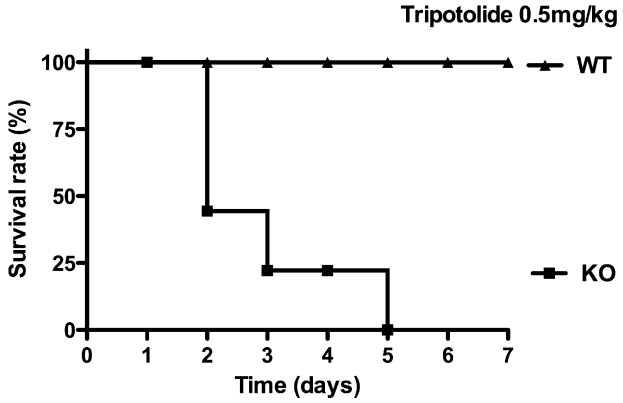

Fig. 1. Survival rate in the KO and WT mice after triptolide treatment. Male KO and WT mice were observed for mortality within 7 days after a single oral dose of triptolide (in saline with 2.5% DMSO) at 0.5 mg/kg, $n = 8$.