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Isoliquiritigenin protects against triptolide-induced hepatotoxicity in mice through Nrf2 activation

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Received February 5, 2016, accepted March 11, 2016

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Pharmazie 71: 394–397 (2016) *doi: 10.1691/ph.2016.6535*

Isoliquiritigenin, a flavonoid found in licorice, has been considered as an antioxidive and hepato-protective agent. Recent studies have shown that a possible mechanism for triptolide-induced hepatotoxicity is related to oxidative damage induced by reactive oxygen species. This study was done to investigate the protection effect of isoliquiritigenin against triptolide-induced hepatotoxicity and the mechanism involved. An acute liver injury model was established by intraperitoneal injection of triptolide (1.0 mg · kg^{-1}) in mice. Different doses of isoliquiritigenin (12.5, 25 and 50 mg · kg^{-1}) were employed as protection. The activities of AST, ALT, ALP and LDH in serum and levels of GSH, GPx, SOD, CAT and MDA in liver tissue were detected. The histopathological changes of liver tissues were observed after HE staining. The protein expression of Nrf2 was detected by western blot. Pretreatment with isoliquiritigenin significantly prevented the triptolide-induced hepatotoxicity indicated by reduced activities of AST, ALT, ALP and LDH. Moreover, isoliquiritigenin pretreatment also prevented from triptolide-induced hepatotoxicity by inhibiting MDA and restoring the levels of GSH, GPx, SOD and CAT. In addition, isoliquiritigenin could attenuate histopathological changes induced by triptolide. Furthermore, the results indicated that isoliquiritigenin pretreatment caused an increase in the protein expression of Nrf2. These results indicated that isoliquiritigenin could protect against triptolide-induced hepatotoxicity via activation of the Nrf2 pathway.

1. Introduction

Licorice, the roots and rhizomes of *Glycyrrhiza* species, is one of the most popular herbal medicines, as it appears in 60% of Traditional Chinese Medicine prescriptions (Xing et al. 2011). Isoliquiritigenin (20, 40, 4-trihydroxychalcone, ISL), a flavonoid found in licorice, has a variety of biological activities, including antioxidant, anti-inflammatory, chemo-preventive, anti-tumor and hepato-protection properties (Chen et al. 2013). Triptolide (TP), a major active ingredient isolated from herbal medicine *Tripterygium wilfordii* Hook f. (TWHF), is considered to hold great promise as a chemotherapeutic agent. However, its potential clinical use is limited due to its severe toxicities especially hepatotoxicity (Li et al. 2014c). It is reported in several studies that a possible mechanism for triptolide-induced hepatotoxicity is related to oxidative damage induced by reactive oxygen species (Mei et al. 2005). The chemical structures of isoliquiritigenin and triptolide are shown in Fig. 1.

It is reported that activation of nuclear erythroid-related factor 2 (Nrf2) could protect against triptolide-induced hepatotoxicity (Li et al. 2014a). Nrf2 is anti-oxidant transcription factor leading a protection against oxidative stress by inducting its target genes (Ma et al. 2013). Under physiological conditions, Nrf2 is present in the cytoplasm binding to the Kelch-like ECH-associated protein1 (Keap1). Under stress conditions, activated Nrf2 dissociates from Keap1, translocates into the nucleus, and interacts with antioxidant response elements. This process induces subsequent expression of numerous downstream genes including heme oxygenase 1 (HO-1), NAD (P) H/quinone oxidoreductase-1, glutamate cysteine ligase, multidrug resistance proteins and glutathione S-transferase (Ma et al. 2012; Shen et al. 2009). Therefore, Nrf2 pathway stimulating agents and related antioxidant genes appear to be a promising approach to detoxification and antioxidation.

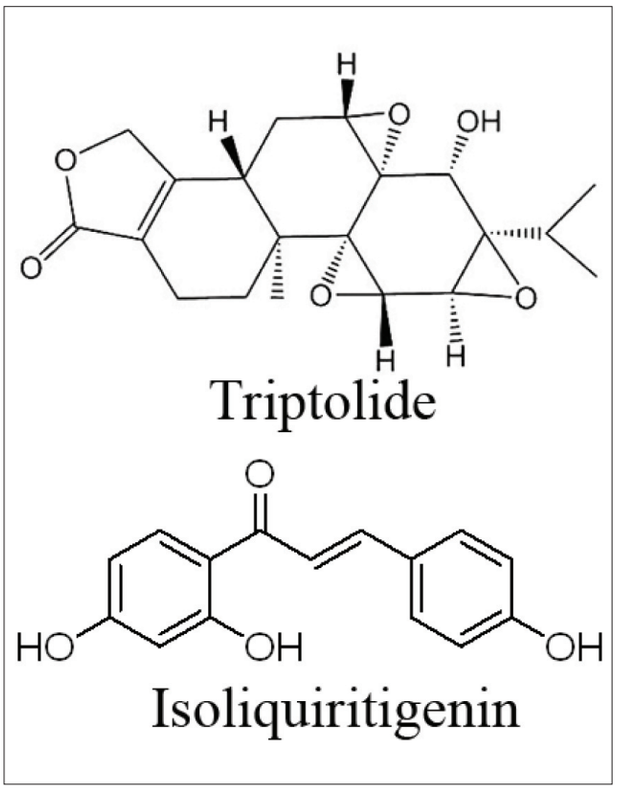

Fig. 1: Chemical structures of triptolide and isoliquiritigenin.

Licorice is often given in combination with TWHF for its toxicity attenuation and efficacy potentiation effect on the treatment of rheumatoid arthritis (Li et al. 2014c; Li et al. 2006). However, details of the hepatoprotection mechanism remain to be elucidated. Previously we have found that isoliquiritigenin (among four compounds derived from licorice) was the most potent Nrf2 pathway inducer in HepG2 (Gong et al. 2015). It might intervene in Nrf2 pathway to induce its target genes, which indicated a novel mechanism for the use of licorice and isoliquiritigenin to lower drug toxicity. To date, whether isoliquiritigenin could protect against triptolide-induced hepatotoxicity has not been investigated *in vivo*. This study was designed to investigate the protective role of isoliquiritigenin on triptolide-induced hepatotoxicity in mice and to examine whether the Nrf2 pathway was involved in this protection. This study is of significant importance for therapeutic intervention in triptolide-induced hepatotoxicity.

2. Investigations and results

2.1. Effect of isoliquiritigenin on triptolide-induced liver injury in ICR mice

To investigate the effect of isoliquiritigenin on triptolide-induced hepatotoxicity, activities of serum ALT, AST, ALP and LDH (Table 1) were measured 24 h after triptolide administration. Triptolide resulted in severe hepatotoxicity as indicated by significantly increased activities of ALT, AST and LDH, whereas ALP was increased but without significant difference. In contrast, this increasing was attenuated by pre-treatment with isoliquiritigenin. In addition, isoliquiritigenin treatment alone had no effects on activities of serum ALT, AST, ALP and LDH compared with the control group.

2.2. Effect of isoliquiritigenin on triptolide-induced oxidative stress in ICR mice

Oxidative stress was quantified through measuring activities of GPx, SOD, CAT and contents of GSH, MDA in liver tissue homogenates (Table 2). Significant reduction in the activities of GPx, SOD, CAT and GSH content were found in the triptolide-treated group compared with those of the control. In contrast, MDA content increased, but there was no significant difference. Isoliquiritigenin ameliorated triptolide-induced hepatotoxicity to some extent as indicated by increased activities of GPx, SOD, CAT and GSH levels and decreased content of MDA. Moreover, high doses of isoliquiritigenin had no effect on the hepatic GSH, GPx, CAT and MDA levels except SOD.

2.3. Effect of isoliquiritigenin on triptolide-induced histopathological changes in ICR mice

Histopathological analysis showed that livers from mice in control and high-dose isoliquiritigenin group appeared smooth, and normal in color without any significant microscopic changes (Fig. 2A and C). However, triptolide-treated mice showed histopathological changes, characterized by fat vacuoles, inflammatory cell infiltration and necrosis (Fig. 2B). Although these findings were also observed in the isoliquiritigenin + triptolide groups (Fig. 2D, E and F), the severity of histopathological lesions were less than those in the triptolide group.

2.4. Effect of isoliquiritigenin on the protein expression of Nrf2

To determine whether isoliquiritigenin elicits its effects by regulating Nrf2, next the protein expression of Nrf2 were determined by western blot. As presented in Fig. 3, triptolide-treated mice showed a slighter decline of Nrf2 compared with the control group. In contrast, the Nrf2 accumulations in the isoliquiritigenin pre-treated group were increased dose-dependently. Moreover, the high-dose isoliquiritigenin treatment alone significantly increased the protein expression of Nrf2 as well.

3. Discussion

Isoliquiritigenin, a flavonoid extracted from licorice, is a potent antioxidant with anti-inflammatory and anti-cancer properties (Vaya et al. 1997; Baba et al. 2002). Previously, we found that isoliquiritigenin was the most potent Nrf2 pathway inducer in HepG2 (Gong et al. 2015) which indicated a novel antioxidative mechanism. Triptolide is among the most powerful anti-inflammatory and immune-modulating natural products ever discovered (Li et al. 2014c). However, the side effects of triptolide, especially its hepatotoxicity, have not been well characterized. The present study focused on the oxidative stress, which has been reported to be implicated in the hepatotoxicity of triptolide (Li et al. 2014a) in HepG2 cells and BALB/C mice.

Consistent with previous reports, triptolide exposure led to obvious hepatotoxicity in livers of ICR mice by decreasing activities of antioxidant enzymes (GPx, SOD and CAT) and content of GSH meanwhile increasing the levels of MDA. Moreover, increased serum activities of AST, ALT, ALP and LDH have been attributed

Table 1: Effect of isoliquiritigenin on serum activities of AST, ALT, ALP and LDH in mice treated with triptolide (n=6).

Group	ALT/U • L^{-1}	AST/U • L^{-1}	ALP/U • L^{-1}	LDH/U • L^{-1}
Con	36.7±12.8	101.1±19.5	95.3±34.1	692.8±156.0
TP	400.3±322.6**	854.0±596.4**	120.0±22.5	2702.9±530.1**
TP+ISL 12.5	388.3±259.3	700.1±359.0	108.9±15.6	1806.3±659.4$^{\#\#}$
TP+ISL 25	124.5±47.7$^{\#\#}$	241.1±144.5$^{\#\#}$	105.1±41.6	1042.6±302.4$^{\#\#}$
TP+ISL 50	54.7±18.5$^{\#\#}$	159.4±31.2$^{\#\#}$	97.4±15.7	958.9±184.4$^{\#\#}$
ISL 50	35.3±4.5	139.5±35.4	94.8±30.0	772.3±115.5**

X ± SD. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs triptolide; Con: control, TP: triptolide, ISL: isoliquiritigenin.

Table 2: Effect of isoliquiritigenin on the hepatic GSH, GSH-PX, SOD, CAT and MDA in mice treated with triptolide (n = 6).

Group	GSH (nmol/mg prot)	GPx (U/mg prot)	SOD (U/mg prot)	CAT (U/mg prot)	MDA (nmol/mg prot)
Con	39.70±1.64	27.09±1.11	4.02±0.38	16.09±0.67	2.41±0.23
TP	31.37±2.85**	18.81±0.46**	2.51±0.35**	14.58±0.17**	3.40±0.44
TP+ISL 12.5	36.06±2.74	21.54±0.81	2.75±0.79	15.29±0.35	2.57±0.32
TP+ISL 25	36.07±2.32	23.57±1.33$^{\#\#}$	3.37±0.18	15.77±0.56$^{\#\#}$	2.35±0.65$^{\#}$
TP+ISL 50	36.50±3.92	22.04±0.93$^{\#}$	3.78±0.49$^{\#\#}$	15.46±0.15$^{\#}$	2.24±0.49$^{\#\#}$
ISL 50	37.66±3.37	25.57±1.44	2.50±0.67**	15.67±0.70	2.85±0.20

X ± SD. $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs triptolide; Con: control, TP: triptolide, ISL: isoliquiritigenin.

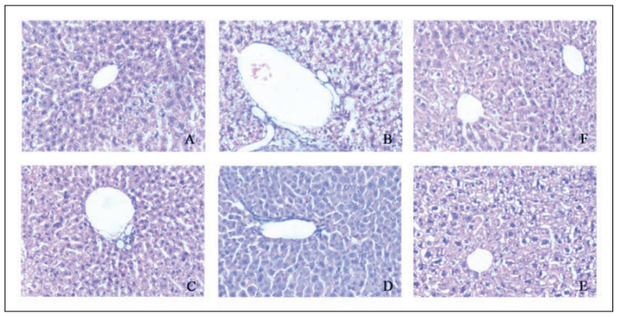

Fig. 2: Histopathological analysis of the liver of mice (Magnification 200×). A: Control; B: TP (1.0 mg • kg^{-1}); C: ISL (50 mg • kg^{-1}); D: TP +ISL (25 mg • kg^{-1}); E: TP+ISL (25 mg • kg^{-1}); F: TP +ISL (50 mg • kg^{-1}). TP: triptolide, ISL: isoliquiritigenin.

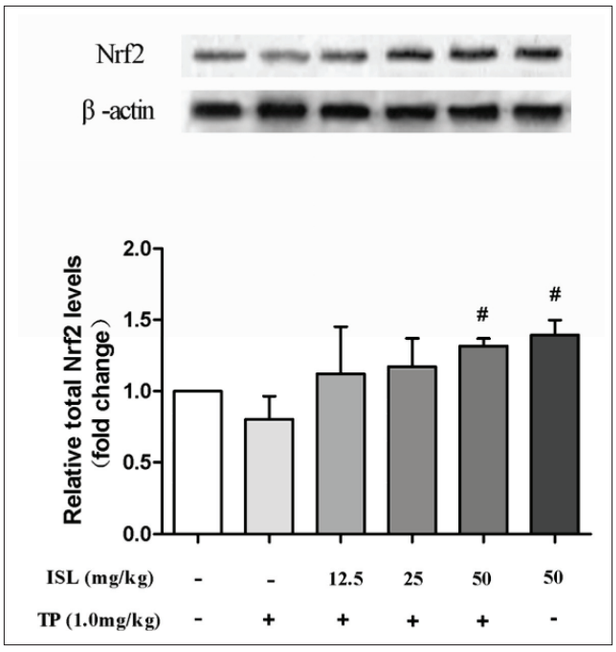

Fig. 3: Effect of isoliquiritigenin on the protein expression of Nrf2 in the liver of mice (n = 3). X ± SD. #P < 0.05 vs triptolide group. TP: triptolide, ISL: isoliquiritigenin.

to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage (Beydilli et al. 2015). GSH and related enzymes constitute the primary defense mechanism against cytotoxicity of xenobiotics, especially oxidative stress. It is vital in detoxification of xenobiotics (Li et al. 2014b). GPx is a crucial selenocysteine-containing enzyme, which catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reducing glutathione and functioning to protect the cells from oxidative damage (Arthur et al. 2000). SOD is regarded as the first line of defense against the detrimental effects of molecular oxygen radicals in cells which protects the cells from superoxide toxicity via removing superoxide free radicals (Beydilli et al. 2015). CAT protects the cells from oxygen-free radical damage by converting hydrogen peroxide formed by the detoxification of superoxide radicals to molecular oxygen and water before it can decompose to form the highly reactive hydroxyl radical (Glorieux et al. 2015; Yu et al. 2015). Additionally, the current results showed that isoliquiritigenin could remarkably increase GSH content, decrease levels of MDA and enhance the activities of GPx, SOD and CAT, thus to protect ICR mice against triptolide-induced hepatotoxicity.

Nrf2, an important endogenous antioxidant transcription factor, has been implicated to play a vital role in oxidative damage induced by various toxic xenobiotics (Enomoto et al. 2001; Rubio et al. 2011). Studies have revealed that Nrf2 knockout mice were more susceptible to chemical hepatotoxins such as acetaminophen (Copple et al. 2008) and CCl_4 (Randle et al. 2008). Furthermore, most Nrf2 inducers including curcumin (Farombi et al. 2008), oleanolic acid (Reisman et al. 2009) are used for chemo-prevention of toxicants-induced hepatotoxicity. These results suggested the potential role of Nrf2 as a therapeutic target to prevent and treat liver injury. As shown in Fig. 1, isoliquiritigenin, containing an α,β-unsaturated carbonyl, is also an electrophilic Michael acceptor with the potential to covalently modify cellular proteins resulting in modulation of Nrf2 pathway (Hajirahimkhan et al. 2015). Another study found that isoliquiritigenin could function as a hepatic protectant by induction of anti-oxidative genes through extracellular signal-regulated kinase-mediated Nrf2 (Park et al. 2015). On the one hand, isoliquiritigenin suppresses lipopolysaccharide-induced inflammatory responses by inhibiting the Keap1, increasing Nrf2 translocation and inducing the mRNA expression of its target genes (Wang et al. 2015). On the other hand, isoliquiritigenin could modulate Nrf2/HO-1 antioxidant axis thus to counteract neuroinflammation and neurodegeneration (Foresti et al. 2013). Although numerous studies demonstrated that isoliquiritigenin could activate the Nrf2 pathway, this current study is the first to combine isoliquiritigenin with triptolide to assess the effect of isoliquiritigenin on reducing triptolide-induced hepatotoxicity.

Collectively, the study has shown that isoliquiritigenin is able to protect ICR mice against triptolide-induced hepatotoxicity. The underlying mechanisms may be due to its antioxidative effect and a Nrf2 pathway activation effect. This study suggests that the powerful antioxidative effects make isoliquiritigenin a novel and potentially advantageous therapeutic agent for treatment of drug-induced hepatotoxicity. Additionally, the Nrf2 pathway may represent a new biological target.

4. Experimental

4.1. Chemicals and reagents

Isoliquiritigenin (purity>99%), and triptolide (purity≥98%) were purchased from On-Road Biotechnology Co. Ltd (Changsha, China). Paraformaldehyde was purchased from Sinopharm Chemical Reagent Co. Ltd (Wuhan, China). Alanine transaminase (ALT) and aspartate transaminase (AST) detection kit was purchased from Wako Pure Chemical Industries (Osaka, Japan); alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) detection kit was purchased from Ningbo medicalsystem biotech Co.Ltd (Ningbo, China). Glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) detection kit was purchased from Nanjing Jiancheng Bioengineering institute (Nanjing, China). Anti-Nrf2 antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Other chemicals were of analytical grade from commercial suppliers.

4.2. Animals and treatments

All animal use procedures were conducted according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China, with the approval of the Ethics Committee in The Experimental Animal Center of the Second Xiangya Hospital. Six to eight week old male ICR mice were housed at 22-25 °C and humidity 50-60% with a 12 h light-dark cycle and had free access to food and water. The mice were randomly divided into six groups (n=6-8): (1) control, (2) triptolide (1.0 mg · kg^{-1}), (3) triptolide + isoliquiritigenin (12.5 mg · kg^{-1}), (4) triptolide + isoliquiritigenin (25 mg · kg^{-1}), (5) triptolide + isoliquiritigenin (50 mg · kg^{-1}), (6) high-dose of isoliquiritigenin (50 mg · kg^{-1}). Mice received either 0.5% (w/v) CMC-Na or isoliquiritigenin once daily for 7 days consecutively. 1 h after the final treatment, mice were treated with triptolide (1.0 mg · kg^{-1}, i.p.. Groups of control animals were given the corresponding vehicles. 6 h after the administration of triptolide, mice were given either CMC-Na or isoliquiritigenin again. In all treated groups, mice were anesthetized 24 h after triptolide injection.

4.3. Serum biochemical parameters analysis

Blood samples were collected and serum was obtained for determination of liver function by measuring the ALT, AST, ALP and LDH using commercially available enzymatic assay kits.

4.4. Antioxidant activities

The extent of oxidative stress was estimated in liver homogenates by measuring the activities of SOD, CAT, GPx and content of GSH, MDA using commercial kits according to the manufacturer's instructions.

4.5. Liver histopathology

Liver samples were taken from the animals and immersed in a 4% (w/v) paraformaldehyde solution for 24 h. After fixation, the specimens were processed through graded alcohols, cleared in turpentine (substitute for xylene) and embedded in paraffin. Then the paraffin blocks were cut and stained with hematoxylin & eosin for morphological evaluation.

4.6. Western blot assay

After treatment with the tested drugs, samples were lysed with RIPA buffer (CW biotech, Beijing, China) and equivalent amounts of protein were separated by 10% SDS-PAGE and transferred to PVDF membranes. After being blocked in 5% non-fat milk in TBST for 1 h at room temperature, the membranes were incubated with the primary antibodies at 4 °C overnight. Subsequently, the immunoblots were then incubated with a secondary antibody at room temperature. The membranes were developed using an electrochemiluminescence (ECL) kit (Advansta, USA) according to the manufacturer's protocol.

4.7. Statistical analysis

Results from the experiments were reported as mean ±standard deviation (SD) and conducted with SPSS 19.0. All data were

analyzed by one-way ANOVA, followed by Tukey's test. Statistical significance was accepted at a P value less than 0.05.

Acknowledgements: This research was supported by the National Natural Science Foundation of China (No. 81202985, 81473411 and 81573686) and the program of China Scholarships Council (No. 201208430206).

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