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Effect of licorice on the induction of phase II metabolizing enzymes and phase III transporters and its possible mechanism

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Licorice has a marked detoxifying effect that can treat drug poisoning and/or relieve adverse effects. However, the exact mechanism of this action is not entirely elucidated, but is believed to be related to the modulation of drug disposition when interacting with other drugs. Additionally, Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a significant role in mediating phase II xenobiotic metabolizing enzymes (XMEs) and phase III transporters. In the present study, we showed that licorice induced the mRNA expression of phase II XMEs UDP-glucuronosyltransferases 1A1 (UGT1A1), glutamate cysteine ligase (GCL), glutathione-s-transferase (GST) and phase III transporters multidrug resistance protein 2 (MRP2), as well as a rapid increase in Nrf2 nuclear accumulation. These findings suggests that licorice may intervene in the Nrf2 signal pathway to induce UGT1A1, GCLC, GST and MRP2, which provide a novel mechanism for the use of licorice to treat drug poisoning and/or relieve adverse effects.

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 (TCM) prescriptions (Xing et al. 2011). Its broad range of applications is partly due to its unique effect on moderating the characteristics of toxic herbs in low dosage. The moderating effects of licorice on other herbs include a marked detoxifying effect that can treat drug poisoning and/or relieve adverse effects (Qiao et al. 2012). The exact mechanism of this action is not entirely elucidated, but is believed to be related to the modulation of drug disposition when interacting with other drugs (Wang et al. 2013).

Two major groups of biotransformation enzymes that metabolize drugs are phase I and phase II xenobiotic metabolizing enzymes (XMEs)(Mandlekar et al. 2006). Some drugs undergo metabolic activation to exhibit toxicity by cytochrome P450 (CYP) enzymes, one major group of phase I XMEs (Kohle and Bock 2007). Compared to the phase I XMEs, phase II XMEs, including UDP-glucuronosyltransferases (UGT), glutamate cysteine ligase (GCL) and glutathione S transferases (GST), are more likely to have protective effects against toxicants (Jana and Mandlekar 2009). In addition to the essential functions of phase II XMEs in drug metabolism, phase III transporter MRPs play a critical role in the excretion of drugs in the body, which is characterized as ATP-dependent export pumps for endo- or xeno-biotic conjugates with glutathione, glucuronate or sulphate (Konig et al. 1999).

It has been demonstrated that Nrf2 plays a significant role in mediating phase II XMEs and phase III transporters (Shen and Kong 2009). Under physiological conditions, Nrf2 is present in the cytoplasm binding to the Kelch-like ECH-associated protein 1 (Keap1) which functions as a negative regulator of Nrf2 by preventing Nrf2 from entering into the nuclear and promoting its proteasomal degradation. Under stress conditions, Nrf2 dissociates from Keap1 and translocates into the nuclear where it dimerizes with small Maf protein and binds to the antioxidant-responsive element (ARE), leading to expression of target genes (Ma and He 2012). Therefore, we provide the hypothesis that licorice may intervene in Nrf2 signal pathway to induce phase II XMEs UGT1A1, GCLC, GST and phase III transporter MRP2, leading to accelerate the elimination of poisonous components and reduce the exposure of poisonous components.

2. Investigations and results

2.1. Effect of licorice extracts on the induction of detoxification genes

To examine whether licorice extracts could regulate the mRNA expression of phase II XMEs and phase III transporters, qPCR analysis was performed. The maximal non-cytotoxic concentration of licorice extract is 120 μg/ml (Tan 2012). As is shown in Fig. 1, the expression of UGT1A1, GCLC, GST and MRP2 was induced by licorice extracts in a dose-dependent manner.

HepG2 cells were treated with the indicated concentrations of licorice (15, 30, 60, 120 μg/ml) for 8 h. The mRNA expressions of UGT1A1 (A), GCLC (B), GST (C) and MRP2 (D) were analyzed by real-time PCR. GAPDH was used as an internal control. The data are represented as the mean ± S.D. from three independent experiments. $*P < 0.05$, $**P < 0.01$ versus Con: control (0.1% DMSO).

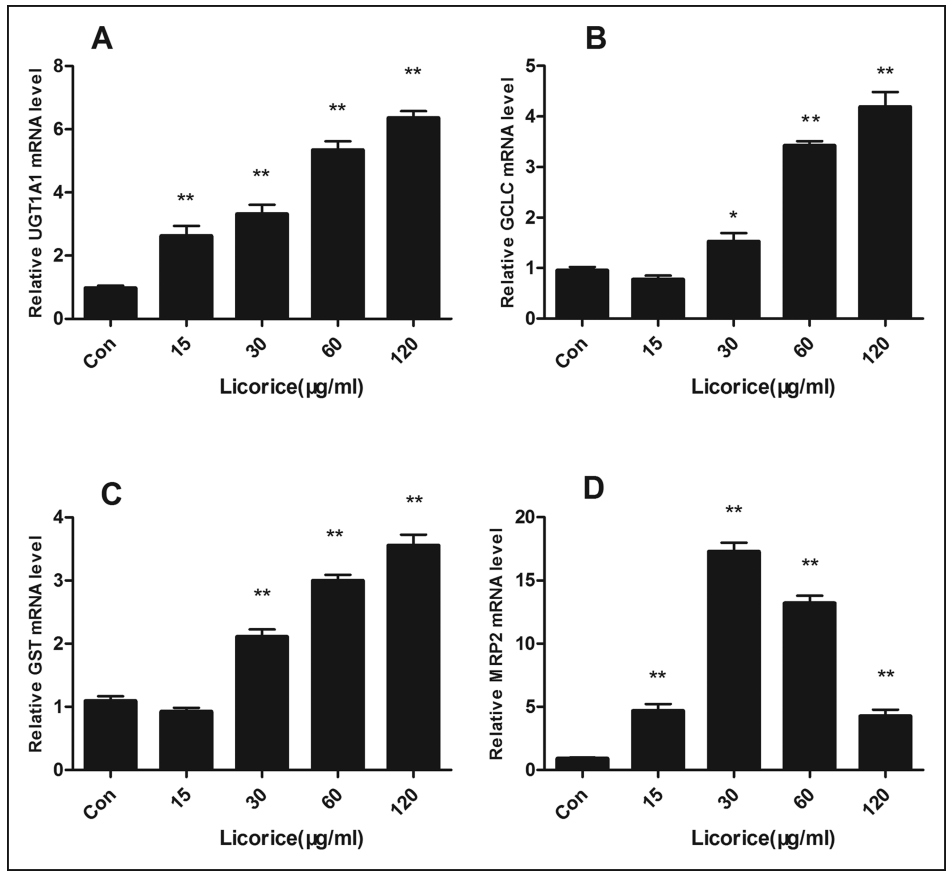

Fig. 1: Effects of licorice on the mRNA expression levels of Nrf2 target genes in HepG2 cells.

2.2. Effect of licorice extracts on the activation of Nrf2

Because Nrf2 nuclear translocation is a key event in the activation of this pathway, we examined Nrf2 nuclear accumulation after licorice extracts treatment to investigate the effect of licorice extracts on Nrf2 activation. The time course of Nrf2 nuclear accumulation is shown in Fig. 2A. Western blot analysis depicted that Nrf2 nuclear levels started to increase at 4 h, reached peak values between 8 h and 10 h after licorice extracts treatment, and declined thereafter. Moreover, HepG2 cells were treated with licorice extracts (15, 30, 60, 120 μg/ml) for 8 h. As shown in Fig. 2B, nuclear levels of Nrf2 were significantly increased at concentration of 30 and 60 μg/ml. Tert-butyl hydroquinone (tBHQ), which is a well-known Nrf2 activator, was used as a positive control.

After being exposed to licorice (30 μg/ml) for the indicated time periods (A) or to the indicated concentrations of licorice (15, 30, 60, 120 μg/ml) for 8 h (B), the nuclear protein lysates were prepared and subjected to Western blot analysis using the indicated antibodies. The PCNA was used as an internal control. For a positive control, HepG2 cells were treated with 20 μM tBHQ for 24 h (A) or 8 h (B).

3. Discussion

Our data demonstrate that licorice induces phase II XMEs UGT1A1, GCLC, GST and phase III transporters MRP2 in HepG2 cells. This induction is probably brought about, at least partly, through its effect on modulating the Nrf2 signal pathway. As a unique drug that reduces toxicity in a multi-herb prescription, the detoxification mechanism of licorice has garnered much attention. Increasing the detoxification rate of the toxic components in our body is an important strategy of protecting cells (Keum 2012). We examined the effect of licorice on the expression of phase II XMEs and phase III transporters using HepG2 cells, which has been employed extensively as the model cell line to study the biology of drug metabolizing enzymes (Huang et al. 2011; Vollrath et al. 2006). Among many phase II XMEs, we focused on UGT1A1, GCLC and GST. UGTs are the key detoxification enzymes, as UGT-catalyzed glucuronidation accounts for up to 35% of phase II reactions (Ishii et al. 2010). UGT1A1 is an important subtype. The presence of the UGT1A1*28 allele, a promoter polymorphism featuring seven TA repeats, dramatically reduces the capacity for glucuronidation and increases the toxicity of irinotecan, a chemotherapeutic drug (Font et al.

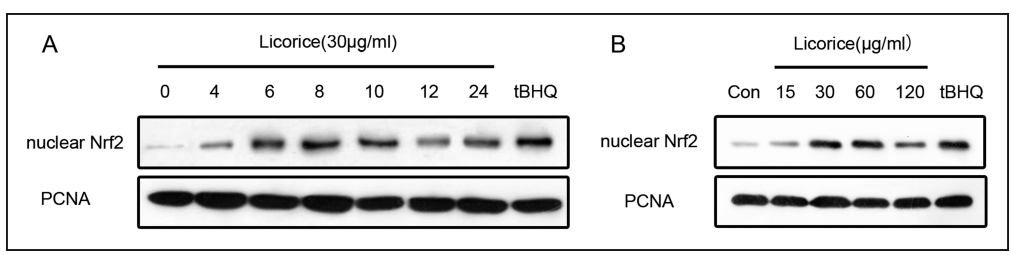

Fig. 2: Effects of Licorice on the nuclear levels of Nrf2 protein.

Table: Human Primers for q-PCR

Gene	Forward	Reverse
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'- GGCATGGACTGTGGTCATGAG -3'
UGT1A1	5'-TAAGTGGCTACCCCAAAACG-3'	5'-TCCAGCTCCCTTAGTCTCCA-3'
GCLC	5'-CTGTTGCAGGAAGGCATTGAT -3'	5'-TTCAAACAGTGTCAGTGGGTCTCT-3'
GST	5'-AGAGGCCATGGTTTGTTGGA-3'	5'-CCTTCAGATTTGGGAAGGCG-3'
MRP2	5'-AAGAGTCTTCGTTCCAGACGC-3'	5'-GGGAATCCACACCAGAACAGT-3'

2003; Gupta et al. 1994). GSH is vital in detoxification of xenobiotics. GCLC is the catalytic subunit of GCL that catalyzes the rate-limiting step in the biosynthesis of GSH. GSTs conjugate glutathione to electrophilic molecules and oxidative metabolites (Lu 2013). Phase III transporter MRP2 plays a key role in cellular protection by removing xenobiotics and metabolites that can accumulate in tissues and lead to toxicity. Our data showed that licorice significantly induced the mRNA expression of UGT1A1, GCLC, GST and MRP2. The activation of these genes conduces to decrease the oxidative burden of the cell by increasing GSH concentrations and general detoxification of chemicals via phase II conjugation reactions as well as efflux transportation.

Nrf2 is presumably the most important transcriptional factor for the cell to deal with oxidative stress and toxicity generated from exposure to exogenous and endogenous chemicals (Niture et al. 2010). Studies have revealed that Nrf2 knockout mice were more susceptible to butylated hydroxyltoluene induced lung injury and acetaminophen induced hepatotoxicity at high dose (Chan and Kan 1999; Enomoto et al. 2001). This protective role of Nrf2 has been attributed in part to its involvement in the induction of phase II XMEs and phase III transporters (Klaassen and Slitt 2005). It has been demonstrated that Nrf2 is essential for ARE-mediated gene activation of UGT1A1, GCLC, GST and MRP2 (Wu et al. 2011; Maher et al. 2007). In the present study, treatment of HepG2 cells with licorice resulted in the activation of Nrf2, as reflected by increased nuclear accumulation of Nrf2, suggesting that the induction of UGT1A1, GCLC, GST and MRP2 by licorice may be through the activation of Nrf2 signal pathway.

In conclusion, our study showed that licorice induces phase II XMEs UGT1A1, GCLC, GST and phase III transporters MRP2 by modulating the Nrf2 signal pathway, which provides new insights into the detoxification mechanism of licorice. However, Nrf2-dependant mechanisms that contribute to licorice-mediated protection from specific drug poisoning require further study.

4. Experimental

4.1. Chemicals and reagents

Licorice extracts were purchased from RuiHong Co. (Xi'an, China). Tert-butyl hydroquinone (tBHQ) and dimethylsulfoxide were purchased from Sigma-Aldrich (St. Louis, Mo). Anti-Nrf2 and PCNA antibodies were purchased from Santa Cruz (Santa Cruz, CA). Other chemicals were of analytical grade from commercial suppliers.

4.2. Cell culture

Human hepatocarcinoma (HepG2) cells obtained from Xiangya cell bank (Changsha, China) were cultured in Dulbecco Modified Eagle Medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) in a 37 °C incubator with 5% CO_2.

4.3. Quantitative real-time PCR analysis

Total mRNA was extracted from cells using TRIZOL reagent (Invitrogen) and equal amounts of RNA were reverse-transcripted to cDNA using Maxima® First Strand cDNA Synthesis Kit (Fermentas, Canada). The cDNA was analyzed using Quantitative real-time PCR with Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas). The level of GAPDH mRNA was used as an internal standard. The primers for real-time PCR analysis are shown in the Table.

4.4. Western blot analysis

Cell extracts were prepared in RIPA buffer (Beyotime, China). Nuclear extracts were prepared using a Nuclear Extract Kit (Thermo, USA) according to the manufacturer's recommendations. Equivalent amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride 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 anti-Nrf2 (1:500) and anti-PCNA (1:2000) at 4 °C overnight. The immunoblots were then incubated with a secondary antibody for 2 h at room temperature. The membranes were developed using an electrochemiluminescence (ECL) kit (Thermo) according to the manufacturer's protocol.

4.5. Statistical analysis

Results from the experiment were reported as means ± S.D. and analyzed using SPSS version 19.0 software. Differences between groups were determined by one-way ANOVA test followed by Tukey's test. The prior level of significance was established at $p < 0.05$ or $p < 0.01$.

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