Journal of Ethnopharmacology 216 (2018) 18–25

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Shikonin upregulates the expression of drug-metabolizing enzymes and drug transporters in primary rat hepatocytes

Chin-Shiu Huang[a,b,1], Haw-Wen Chen[c,1], Tzu-Yu Lin[c], Ai-Hsuan Lin[c], Chong-Kuei Lii[a,c,*]

[a] Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan
[b] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan
[c] Department of Nutrition, China Medical University, No. 91, Hsueh-Shih Road, Taichung 404, Taiwan

ARTICLE INFO

Chemical compound studied in this article:
Shikonin (PubChem CID:479503)

Keywords:
Shikonin
Drug-metabolizing enzymes
Drug transporters
Aryl hydrocarbon receptor
NF-E2-related factor

ABSTRACT

Ethnopharmacological relevance: Shikonin, a naphthoquinone pigment abundant in the root of the Chinese herb *Lithospermum erythrorhizon*, has been widely used to treat inflammatory diseases for thousands of years. Whether shikonin changes drug metabolism remains unclear.

Aim of the study: We investigated whether shikonin modulates the expression of hepatic drug-metabolizing enzymes and transporters as well as the possible mechanisms of this action.

Materials and methods: Primary hepatocytes isolated from Sprague-Dawley rats were treated with 0–2 μM shikonin and the protein and mRNA levels of drug-metabolizing enzymes and transporters as well as the activation of aryl hydrocarbon receptor (AhR) and NF-E2-related factor 2 (Nrf2) were determined.

Results: Shikonin dose-dependently increased the protein and RNA expression of phase I enzymes, i.e., cytochrome P450 (CYP) 1A1/2, CYP3A2, CYP2D1, and CYP2C6; phase II enzymes, i.e., glutathione *S*-transferase (GST), NADP(H) quinone oxidoreductase 1 (NQO1), and UDP glucuronosyltransferase 1A1; and phase III drug transporters, i.e., P-glycoprotein, multidrug resistance-associated protein 2/3, organic anion transporting polypeptide (OATP) 1B1, and OATP2B1. Immunoblot analysis and EMSA revealed that shikonin increased AhR and Nrf2 nuclear contents and DNA binding activity. AhR and Nrf2 knockdown by siRNA attenuated the ability of shikonin to induce drug-metabolizing enzyme expression. In addition, shikonin increased p38, JNK, and ERK1/2 phosphorylation, and inhibitors of the respective kinases inhibited shikonin-induced Nrf2 nuclear translocation.

Conclusions: Shikonin effectively upregulates the transcription of CYP isozymes, phase II detoxification enzymes, and phase III membrane transporters and this function is at least partially through activation of AhR and Nrf2. Moreover, Nrf2 activation is dependent on mitogen-activated protein kinases.

1. Introduction

Herbal medicine is becoming popular as a component of complementary and alternative medicine worldwide (Enioutina et al., 2017). It is therefore important to understand the effects of herb products and herb phytochemicals on the metabolism-based herb-drug, herb-herb, and herb-food interactions. In fact, metabolic interactions between herbs and drugs are common, and some can lead to therapeutic failure or toxicity (Shi and Klotz, 2012; Sørensen, 2002). For instance, grapefruit juice, St. John's wort (*Hypericum perforatum*), chamomile (*Matricaria recutita*), peppermint (*Mentha piperata*), dandelion (*Taraxacum officinale*), and ginseng (*Zingiber officinale* Rosc.) have been reported to inhibit or stimulate the activity of cytochrome P450s (CYPs), phase II conjugating enzymes, and drug transporter proteins, which can in turn alter the efficacy of immunosuppressive, anticancer, anti-hyperlipidemic, anti-retroviral, anti-depressant, and anti-coagulative drugs (Nowack, 2008).

Lipophilic foreign compounds are transformed into hydrophilic metabolites via drug metabolism, with the result being the acceleration of excretion of these compounds (Xu et al., 2005). For this goal to be

Abbreviations: AhR, aryl hydrocarbon receptor; AP1, activating protein 1; ARE, antioxidant-response element; CAR, constitutive androstane receptor; CYP, cytochrome P450; DREs, dioxin-responsive elements; EMSA, electromobility gel shift assay; FBS, fetal bovine serum; GST, glutathione transferase; ITS, insulin-transferrin-selenium; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinases; MRP, multidrug resistance-associated proteins; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid-derived 2-related factor 2; OATP, organic anion transporting polypeptide; P-gp, p-glycoprotein; PXR, pregnane X receptor; UGT, UDP-glucuronosyltransferases

* Corresponding author.
E-mail address: cklii@mail.cmu.edu.tw (C.-K. Lii).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jep.2018.01.026
Received 12 August 2017; Received in revised form 17 January 2018; Accepted 19 January 2018

reached efficiently, multiple enzymes and membrane transporters are required to work in cooperation. According to the roles of these proteins, they are grouped into three phases: phase I (enzymes responsible for adding or exposing –OH, –SH, –NH2, or –COOH groups on xenobiotics), phase II (enzymes responsible for conjugating xenobiotics with small water-soluble and polar molecules), and phase III (transporters responsible for xenobiotic uptake and exportation). CYPs are the most important phase I enzymes and catalyze the oxidation, reduction, and hydrolysis of many drugs, industrial chemicals, environmental pollutants, carcinogens, and herbal components as well as endogenous compounds (Xu et al., 2005). More than 50 CYP isozymes have been identified in humans (Danielson, 2002). The most active CYPs for drug metabolism are CYP1A2, CYP2C8/9/19, CYP2D6, CYP2E1, and CYP3A4/5 (Danielson, 2002; McGraw and Waller, 2012). Among them, CYP3A4/5 is the most abundantly expressed in the liver and small intestine and is involved in the metabolism of about 34% of clinical drugs (Gelatin et al., 2008; McGraw and Waller, 2012). Phase II enzymes are composed of sulfotransferases, UDP-glucuronosyltransferases (UGTs), glutathione transferases (GSTs), and amino acid conjugases, which catalyze the conjugation reactions with sulfate, glucuronic acid, glutathione, and glycine, respectively, to foreign molecules or metabolites generated by the phase I enzymes (Xu et al., 2005). Following the phase I and II reactions, these more polar metabolites are excreted through phase III transporters, such as p-glycoprotein (P-gp, also named multidrug resistance protein 1), multidrug resistance-associated proteins (MRPs), and breast cancer resistance proteins (BCRP/ABCG2). In addition, a number of transporters, such as organic anion transporter proteins (OATPs), organic cation transporters, and peptide transporters, mediate the uptake of organics anions, cations, and peptides (Klaassen and Aleksunes, 2010).

The activity and gene transcription of drug-metabolizing enzymes and drug transporters are susceptible to modulation by many chemicals, including environmental pollutants, drugs, and microbial products, as well as by herb and food components (Guengerich, 1995; Waxman, 1999; Wu et al., 2013). Many transcription factors including ligand-activated nuclear receptors such as pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), and constitutive active receptor (CAR) and phosphorylation-activated transcriptional factors such as nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and activating protein 1 (AP1) have been demonstrated to act coordinately in promoting the transcription of genes encoding drug metabolism proteins (Xu et al., 2005).

Shikonin, a naphthoquinone pigment, is one of the main active components isolated from the dried root of *Lithospermum erythrorhizon* (Sieb. et Zucc, Boraginaceae). *L erythrorhizon* has been widely used for the treatment of burns, macular eruption, sore throat, hemorrhoids, dermatitis, and other inflammatory and infectious diseases in traditional Chinese medicine for thousands of years (Chen et al., 2002). Over the past decades, shikonin has been shown to possess antioxidant, anti-inflammatory, antithrombotic, antimicrobial, wound healing, and antitumor properties (Andújar et al., 2013). Shikonin protects against oxidized low-density lipoprotein-induced endothelial damage by up-regulating antioxidant defense and suppressing NFκB-mediated intercellular adhesion molecule 1 and E-selectin expression (Huang et al., 2013). Shikonin induces apoptosis of human pancreatic cancer cells Bx-PC3, PANC-1, and AsPC-1 and inhibits pancreatic tumor growth by suppressing the NF-κB-dependent pathway (Wang et al., 2014). Recently, shikonin was reported to enhance cisplatin-induced human colon cancer cell killing in vitro and in vivo (He et al., 2016). Although the ethnopharmacological use and clinical application of shikonin has increased, whether this naphthoquinone changes drug metabolism is not clearly understood. In this study, we examined the modulatory potency of shikonin on the expression of CYP isozymes, UGT, GST, NQO-1, and drug transporter proteins in primary rat hepatocytes. We also investigated the possible mechanisms underlying these actions.

2. Materials and methods

2.1. Materials

Shikonin (purity > 97%) was obtained from Calbiochem (Darmstadt, Germany) and was delivered to the cells using dimethyl sulfoxide (Merck, Germany). RPMI 1640 and penicillin-streptomycin solution were from Gibco Laboratory (Grand Island, NY), fetal bovine serum (FBS) was purchased from HyClone (Logan, UT), and insulin-selenium-selenium premix (ITS^+) was from Collaborative Biomedical Products (Bedford, MA). Polyclonal antibodies against Akt (#9272), phospho-Akt (S473/T308, #9271), p38 (#9212), phospho-p38 (#9211), ERK1/2 (#9102), phospho-ERK1/2 (#9101), and PARP (#9542) were obtained from Cell Signaling Technology (Boston, USA); OATP2B1 (#46821) was from GENE Tex (San Antonio, Texas); UGT1A1 (sc-27419), NQO1 (sc-16464), Nrf2 (sc-722), and clathrin (sc-6579) were from Santa Cruz Biotechnology (Santa Cruz, CA); MRP2 (#251463) was from Abbiotec (San Diego, CA); and CYP1A1 (AB1247), CYP3A1 (AB1253), CYP3A2 (AB1276), CYP2D1 (AB1271), and OATP1B1 (AB3572P) were from Chemicon (Temecula, CA). Monoclonal antibodies against CYP1A2 (MAB10035) and CYP2C6 (MAB10040) were purchased from Chemicon, P-glycoprotein (P-gp, #c219) was from Calbiochem (Darmstadt, Germany), π form of GST (PGST, #610718) was from BD Biosciences (San Jose, CA), and AhR (ab2770) and MRP3 (ab3775) were from Abcam (Cambridge, UK). HEPES, protease inhibitors cocktail, leupeptin, and all other chemicals were purchased from Sigma (St. Louis, MO) unless specified otherwise.

2.2. Cell isolation and culture

Male Sprague-Dawley rats obtained from the Bio LASCO Experimental Animal Center (Taipei, Taiwan) were used for hepatocyte isolation at the age of 6–8 weeks. The animal experiments in this study were approved by the Institutional Animal Care and Use Committee of China Medical University. Hepatocytes were isolated by a two-step collagenase perfusion method as described earlier (Huang et al., 2014). The isolated hepatocytes were suspended in RPMI 1640-based culture medium (pH 7.4) containing 10 mM HEPES, 2.6 mM sodium bicarbonate, 1 μM dexamethasone, 100 units/mL penicillin, 1 μg/mL streptomycin, and 2.5% FBS at a density of 5×10^5 cells/mL. A total of 1.2×10^6 cells was planted on 3-cm plastic culture dishes precoated with type I rat tail collagen and incubated at 37 °C in a 5% CO_2 humidified incubator. After 4 h, hepatocytes were changed to fresh RPMI-1640-based culture medium with 1% ITS^+ substitute for FBS. Twenty-four hours after isolation, cells were treated with various concentrations of shikonin for the times indicated. Dimethyl sulfoxide was used as a vehicle for shokonin and the final concentration of dimethyl sulfoxide was 0.1%.

The cytotoxicity of shikonin was determined by (3-(4,5)-dimethylthiazol(2y1))-2,5-di-phenyltetrazolium(3-(4,5)-dimethylthiazol (2y1) − 2,5-diphenyltetrazolium bromide (MTT) assay. Incubation of shikonin up to 2 μM for 24 h did not change cell viability (> 90%).

2.3. Western blotting analysis

Cells were washed twice with cold PBS and were then scraped with a lysis buffer containing 7.4 mM K_2HPO_4, 2.6 mM KH_2PO_4, 154 mM KCl, and protease inhibitor cocktail. Cell homogenates were then sonicated and centrifuged at 10,000 × *g* at 4 °C for 10 min. The supernatants were treated as a cellular protein. The protein contents were analyzed by use of the Coomassie Plus protein assay kit (Pierce, Rockford, IL). For the membrane preparation, a ProteoExtract® subcellular Proteome Extraction kit (Calbiochem, Darmstadt, Germany) was used according to the manufacturer's instructions. Equal amounts of proteins were electrophoresed in a SDS-polyacrylamide gel, and proteins were then transferred to polyvinylidene fluoride membranes.