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Research Paper

Reversing effects of lignans on CCl_4-induced hepatic CYP450 down regulation by attenuating oxidative stress

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ABSTRACT

Oxidative stress has been proved to be a critical reason of regulating CYP450s under hepatic injury status. The study was aimed to investigate the effect of pretreatment of schisandra lignan extracts (SLE) and dimethyl diphenyl bicarboxylate (DDB) on expressions and activities of the main liver P450 isoenzymes in CCl_4 induced liver injury rats and their anti-oxidative effects on both CCl_4 induced liver injury rats and a CCl_4 induced HepG2 cell injury model. Acute experimental liver injury induced by CCl_4 caused drastically decreasing activities of the main liver P450 isoenzymes such as CYP1A2, CYP2C6, CYP2E1 and CYP3A2, as well as their protein expressions. Pretreatment of SLE (500 mg/kg) and DDB (200 mg/kg) twice a day for three days significantly decreased the losses of activities of CYP1A2, CYP2C6, CYP2E1 and CYP3A2. Similar results were observed in protein expressions. In addition, in the CCl_4 induced HepG2 cells injury model and the CYP3A activity level correlated well with ROS level in several ingredients of SLE treated groups, especially in γ-schisandrin group. These results indicated that the reversion of P450 after SLE/DDB treatment were, on one hand, due to hepatoprotective effects of these lignans on livers; on the other hand, due to their regulation of P450 through anti-oxidative effect and γ-schisandrin might be the most powerful ingredient of SLE. Also, there might be potential interactions between SLE or DDB and co-administered medicines and it is necessary to adjust the dosage of co-administrated medicines in clinical medication of liver disease.

1. Introduction

Oxidative stress has been proved to be elevated in many conditions including liver diseases, rheumatoid arthritis, inflammatory bowel diseases and cancer (Kulmatycki and Jamali, 2005) and is critical reason for regulation of the expression of several P450 isoforms under such pathophysiological conditions, especially under liver injury. A substantial body of evidence has demonstrated the pivotal role of oxidative stress in the transcriptional regulation of CYP1A1 (Whitlock, 1999), CYP3A4 (Guengerich, 1999), CYP2B and CYP4A (Morgan, 1997; Waxman, 1999; Aitken et al., 2006) and reactive oxygen species (ROS) generation triggered by H_2O_2 has been proved to decrease CYP1A1 expression in negative feedback way that limits CYP1A1 induction and the related toxicity (Morel et al., 1999; Barouki and Morel, 2001). Also, CYP2E1 is another important enzyme in generating reactive species at the active site which might directly inactivate CYP2E1 and enhance its degradation (Dai and Cederbaum, 1995).

Liver injury, induced by various pathological factors such as hepatic virus, chemical hepatotoxins, and fatty livers, is a widespread pathology which in most cases involves oxidative stress and always leads to the down-regulation of many drug metabolizing enzymes (DMEs) (Ota et al., 1975; Badger et al., 1996). Carbon tetrachloride is one of the most classic xenobiotic to cause hepatotoxicity (Kodavanti et al., 1989). Reductive dehalogenation of CCl_4 by the P450 enzyme system to the highly reactive trichloromethyl radical initiates the process of lipid peroxidation which is considered to be the most important mechanism in the pathogenesis of liver damage induced by CCl_4 (Demirdag et al., 2004). CYP2E1, CYP2B and CYP3A are the major isozymes involved in bioactivation of CCl_4 and subsequent production of free radicals (Recknagel et al., 1989; Weber et al., 2003). However, little study

Abbreviations: SLE, schisandra lignans extract; DDB, dimethyl diphenyl bicarboxylate; CCl4, carbon tetrachloride; DMEs, drug metabolizing enzymes(DMEs); P450, cytochrom P450; NADP+, β-nicotinamide adenine dinucleotide phosphate; LC-MS, liquid chromatography mass spectrometry; MDZ, midazolam; S.D., standard deviation; Clint, intrinsic clearance; TBST, tris-buffered saline/tween 20; PMSF, phenylmethyl sulfonylfluoride; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electropheresis; TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; GST, glutathione S-transferase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescein diacetate

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has been taken to clarify whether there were regulation effects of hepatoprotective agents on P450 isoenzymes activities and protein expressions during CCl_4 induced oxidative stress.

Fruits of *Schisandra chinensis* (Turcz.) Baill. have been used in China to treat viral and chemical hepatitis clinically for a long time as an alternative medicine because of its capability to protect the liver from injuries induced by hepatotoxins (Lu and Liu, 1991; Hancke et al., 1999; Pan et al., 2009). Dozens of structurally similar lignans such as schizandrin A, B and C, schizandrol A and B, schisantherin A and B have been identified from the extract of *schisandra chinensis* (Opletal et al., 2004; Huang et al., 2007) and were proven to be responsible for the hepatoprotection by lowering the elevated serum transaminases based on some chemical induced liver injury model such as CCl_4 which was a widely used chemical hepatotoxin causing an acute liver failure (ALF) (Ip and Ko, 1996; Ip et al., 1996). The effects of schisandra lignans extract (SLE) on P450 isoenzyme expression and activity have been paid a lot of attention. Schisandra has been proved to be a strong inducer of CYP3A4 and 2C9 through PXR in normal hepatocyte cultures (Mu et al., 2006) and dimethyl diphenyl bicarboxylate (DDB) showed strong induction on liver microsomal CYP2B1 in normal rats (Li et al., 1992). Several constituents in SLE and DDB were identified and characterized as potent P450 isoenzymes inhibitors, in vitro studies showed a crucial role played by SLE and DDB on P450 isoenzymes (Kim et al., 2001; Iwata et al., 2004). SLE and DDB could also regualte P450 isoenzymes under hepatic injury status caused by thioacemide (Xie et al., 2012). Our lab has found that SLE and DDB can enhance the metabolism capability of schisandra lignans in CCl_4 induced acute hepatic injury rats (Xie et al., 2010) which clued on the potential regulation of P450 isoenzymes during CCl_4 induced liver pathology while its mechanism still needed further investigation.

Based on the former studies, we hypothesized that SLE and DDB, functioned as hepatoprotective agents, might have regulatory effects on hepatic CYP450 isoenzymes expressions and activities in liver injury model rats through anti-oxidant stress pathway which may be a counterevidence that oxidative stress was the direct reason resulting in P450s down-regulation. Thus, this study was mainly focused on: (1) elucidating the effect of SLE and DDB on oxidative status on both rats and HepG2 cells treated with CCl_4; (2) evaluating the regulating effects of SLE and DDB on the major isozymes of P450s in CCl_4 induced hepatic injury rats and in HepG2 injured model caused by CCl_4; (3) correlating CYP3A4 activity with oxidative stress biomarker to explore the potential relationship between anti-oxidative stress effect and P450 regulation of lignans, proving that medication on oxidative stress might be an important pathway to regulate P450.

2. Methods and materials

2.1. Materials

The ethanol extract of *Schisandra chinensis Bail* was purchased from Nanjing Qingze Pharmaceuticals Company (Jiangsu, China). The drug substance of dimethyl diphenyl bicarboxylate DDB was purchased from Hangzhou Dengyun Pharm & Tech Co., Ltd. pharmacy (Zhejiang, China). Midazolam, chlorzoxazone, diclofenac, phenacetin, acetaminophen γ-schisandrin, schisandrin and schisantherin A were obtained from national institute for the control of pharmaceutical and biological products (Beijing, China). Resorufin, pentoxyresorufin, 4'-hydroxydiclofenac, 4-hydroxymidazolam, 6-hydroxychlorzoxazone, MTT and DCFH-DA were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Glucose 6-phosphate, $NADP^+$, and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile, methanol, and ethyl acetate were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore Corporation, Billerica, MA). Carbon tetrachloride was supplied by Chemical Agent Company of Shanghai (Shanghai, China). Radio immunoprecipitation assay analysis buffer, phenylmethanesulfonyl fluoride, and SDS-PAGE sample loading buffer were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Polyvinylidene difluoride membranes were obtained from Millipore (Shanghai, China). Rabbit anti-rat CYP3A2 polyclonal antibody, rabbit anti-rat CYP2B1/2 polyclonal antibody, rabbit anti-rat CYP2E1 polyclonal antibody and mouse anti-rat CYP1A2 polyclonal antibody were from Chemicon Corporation (U.S.A). Mouse anti-rat CYP2C6 polyclonal antibody was from Santa Cruz (U.S.A). Mouse anti-rat β-actin polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Boster Biological Technology, Ltd. (Wuhan, China). Enhanced chemiluminescence kit was purchased from Pierce Chemical Company (Rockford, USA).

2.2. Animals and treatments

Male Sprague–Dawley rats (180–220 g) were acclimatized to the facilities for a week, and then fasted with free access to water for 12 h prior to each experiment. In each test group, six rats were employed in the present study. Group I was served as normal control, Group II was CCl_4-induced liver injury control and Groups III and IV were CCl_4-induced liver injury rats pretreated with SLE and DDB at a dose of 500 mg/kg and 200 mg/kg body weight for seven times with an interval of 12 h, respectively. On day 4, half an hour after the seventh dose, CCl_4 (2 ml/kg, 1:1 (v/v) mixture of CCl_4 and corn oil, i.p.) was administrated for Groups II–IV. Normal control received an equal amount of corn oil instead of CCl_4. 24 h later, rats were sacrificed for their serum and livers. All the experiments have been carried out in accordance with the internationally accepted guide for the care and use of laboratory animals.

2.3. Cell

The male human Caucasian hepatocyte carcinoma (HepG2) cell lines were purchased from the American Type Culture Collection (Bethesda, MD, USA) and were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco BRL, Paisley, UK) and 1% antibiotic–antimycotic (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B) in 5% CO_2 at 37 °C.

2.4. Preparation of microsomes and enzyme assays

Rat liver microsomes were prepared as described previously (Kamataki and Kitagawa, 1974; Kaul and Novak, 1987) and protein concentrations were measured by the method of Lowry et al. (1951). The P450 enzymatic activities were characterized based on the following reactions: phenacetin O-deethylation, pentoxyresorufin O-dealkylation, diclofenac 4-hydroxylation, chlorzoxazone 6-hydroxylation, and midazolam 4-hydroxylation activities were determined as described previously with minor modifications (Qiu et al., 2008). 200 μl incubation mixture for a regular assay procedure was prepared with 0.2 mg/ml liver microsomes, 10 mM glucose 6-phosphate, 0.5 mM NADP, 10 mM $MgCl_2$, 1 unit of glucose 6-phosphate dehydrogenase, 100 mM phosphate buffer, pH 7.4, and each probe substrate of P450 enzymes. Chlorzoxazone was previously dissolved in methanol, whose final concentration in incubation mixtures was less than 1% v/v, and the others were previously dissolved in water. Substrate concentrations used were: phenacetin, 5–400 μM; pentoxyresorufin, 1–20 μM; diclofenac,