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Glycyrrhizin, silymarin, and ursodeoxycholic acid regulate a common hepatoprotective pathway in HepG2 cells

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ABSTRACT

Background: Glycyrrhizin, silymarin, and ursodeoxycholic acid are widely used hepatoprotectants for the treatment of liver disorders, such as hepatitis C virus infection, primary biliary cirrhosis, and hepatocellular carcinoma.
Purpose: The gene expression profiles of HepG2 cells responsive to glycyrrhizin, silymarin, and ursodeoxycholic acid were analyzed in this study.
Methods: HepG2 cells were treated with 25 μM hepatoprotectants for 24 h. Gene expression profiles of hepatoprotectants-treated cells were analyzed by oligonucleotide microarray in triplicates. Nuclear factor-κB (NF-κB) activities were assessed by luciferase assay.
Results: Among a total of 30,968 genes, 252 genes were commonly regulated by glycyrrhizin, silymarin, and ursodeoxycholic acid. These compounds affected the expression of genes relevant various biological pathways, such as neurotransmission, and glucose and lipid metabolism. Genes involved in hepatocarcinogenesis, apoptosis, and anti-oxidative pathways were differentially regulated by all compounds. Moreover, interaction networks showed that NF-κB might play a central role in the regulation of gene expression. Further analysis revealed that these hepatoprotectants inhibited NF-κB activities in a dose-dependent manner.
Conclusion: Our data suggested that glycyrrhizin, silymarin, and ursodeoxycholic acid regulated the expression of genes relevant to apoptosis and oxidative stress in HepG2 cells. Moreover, the regulation by these hepatoprotectants might be relevant to the suppression of NF-κB activities.

Introduction

Liver cancer is the second most common cause of cancer death worldwide, causing about 746,000 deaths in 2012. The prognosis of liver cancer is very poor and the estimated incidence of new cases is 782,000 in the less developed regions in 2012 (Ferlay et al. 2015). Several studies illustrate that constitutive nuclear factor-κB (NF-κB) activity plays a central role in the hepatic neoplastic progression through the upregulation of anti-apoptotic genes (Kucharczak et al., 2003). Moreover, the inhibition of NF-κB activation in hepatocytes retards and reduces the development of hepatocellular carcinoma in mice (DiDonato et al. 2012). Therefore, the inhibition of NF-κB activation might be an effective strategy to treat liver cancers.

Glycyrrhizin, the triterpenoid saponin from *Glycyrrhiza glabra* L. roots (licorice), consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid. Glycyrrhizin exhibits various pharmacological effects, such as anti-inflammatory and protective effects in liver (Li et al. 2014). Therefore, glycyrrhizin analogs, such

Abbreviations: NF-κB, nuclear factor-κB; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; sva, surrogate variable analysis; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; qPCR, quantitative real-time polymerase chain reaction; RQ, relative quantitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC9, histone deacetylase 9; MCM3AP, minichromosome maintenance deficient 3 associated protein; Cerebral, cell region-based rendering and layout; RLU, relative luciferase unit; IL, interleukin; FGF10, fibroblast growth factor 10; COL2A1, collagen type 2 alpha 1; SPIB, Spi-B transcription factor; PRKAB1, protein kinase AMP-activated beta 1; ETHE1, ethylmalonic encephalopathy 1; BID, BH3 interacting domain death agonist; SOD2, superoxide dismutase 2; MAP3K7, mitogen-activated protein kinase kinase kinase 7; tBID, truncated BID; TNF, tumor necrosis factor; ROS, reactive oxygen species.

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as magnesium isoglycyrrhizinate and stronger neo-minophagen C, are effective and safe for the treatment of patients with chronic liver disease and liver dysfunction (Mori et al. 1990; Mao et al. 2009).

Silymarin is a flavonolignan complex from *Silybum marianum* (L.) Gaertn. fruits. Silymarin comprises a number of flavonolignans, including silibinin (silybin A and silybin B), isosilybin A and B, silychristin A and B, silydianin, and other phenolic compounds (Wu et al. 2009). Silymarin exhibits anti-inflammatory and immunomodulatory effects and thus promotes the health of livers (Polyak et al. 2013). In addition, silymarin-type drugs like legalon have been used for the treatment of acute hepatitis and nonalcoholic fatty liver disease in patients (El-Kamary et al. 2009; Loguercio et al. 2012).

Ursodeoxycholic acid, a hydrophilic stereoisomer of chenodeoxycholic acid, is a major component of Chinese black bear's bile (Ohtsuki et al., 1992). Ursodeoxycholic acid is used to treat chronic cholestatic liver diseases, such as primary biliary cirrhosis and primary sclerosing cholangitis (Chapman 2009; Lindor et al. 2009). Moreover, some evidences indicate that ursodeoxycholic acid decreases the levels of alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyl transpeptidase in patients with chronic hepatitis C and protects livers from against methotrexate-induced toxicity (Omata et al. 2007; Uraz et al. 2008).

Few reports have evaluated the genomic alterations elicited by glycyrrhizin, silymarin, and ursodeoxycholic acid. For examples, *Glycyrrhiza glabra* root extract induces the proliferation of MCF-7 cells by activating extracellular signal-regulated kinases 1/2 and Akt pathways (Dong et al., 2007). Treating hepatocytes with ursodeoxycholic acid shows that ursodeoxycholic acid affects the expression of genes directly involved in cell cycle and apoptotic events, and the E2F-1/p53/apoptotic protease activating factor-1 pathway seems to be the target of ursodeoxycholic acid (Castro et al. 2005). In this study, we treated hepatocytes with non-cytotoxic concentrations of glycyrrhizin, silymarin, and ursodeoxycholic acid, and analyzed the gene expression profiles by microarray. The gene expression profiles were further compared to evaluate the different and the common pathways regulated by these compounds.

Materials and methods

Cell culture

The human hepatoma cell line (HepG2) was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). Recombinant HepG2/NF-κB cell, which carried the NF-κB-driven luciferase genes, was constructed as described previously (Hsiang et al. 2009). HepG2 cells and HepG2/NF-κB cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 μg/ml streptomycin, and 100 unit/ml penicillin in a humidified incubator at 37 °C with 5% CO_2.

Chemicals

Glycyrrhizin (purity $\geq$ 95%), silymarin, and ursodeoxycholic acid (purity $\geq$ 99%) were purchased from Sigma (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 200 mM, and stored at −30 °C. Silymarin (product number 254924) is a mixture of toxifolin (4%), silichristin (27.9%), silidianin (2.9%), silybin A (19.3%), silybin B (31.3%), isosilybin A (8.2%), and isosilybin B (2.3%). MG-132, a NF-κB inhibitor, was purchased from Santa Cruz (Dallas, TX, USA) and dissolved in DMSO to a final concentration of 50 mM. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2).

Total RNA isolation

HepG2 cells (2×10^6 cells, passage number 38) were seeded in a 25-cm^2 flask and incubated at 37 °C for 24 h. Cells were then treated with 5 ml of culture medium containing 0.125‰ DMSO (solvent control) or μM hepatoprotectants, and incubated at 37 °C for another 24 h. Total RNA was extracted from cells treated with or without compounds by RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was quantified using the spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and further evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA sample with an RNA integrity number greater than 8.0 was accepted for microarray analysis.

Microarray analysis

Microarray analysis was performed as described previously (Lo et al. 2013; Ho et al. 2014). Briefly, fluorescent RNA targets were prepared from 5 μg of total RNA samples using the MessageAmp™ aRNA kit (Ambion, Austin, TX, USA) and Cy5 dye (Amersham Pharmacia, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Human Whole Genome OneArray™ (Phalanx Biotech Group, Hsinchu, Taiwan). Number of replicates was three. After an overnight hybridization at 50 °C, non-specific targets were washed away and the array was scanned by an Axon 4000 scanner (Molecular Devices, Sunnyvale, CA, USA). Spots with a signal-to-noise ratio $>$ 0 or control probes were selected and normalized by the R program of the limma package (Smyth and Speed 2003). We used surrogate variable analysis (sva) to capture the heterogeneity of expression caused by any variation and to improve the accuracy and reproducibility in analyzing gene expression levels (Leek and Storey 2007). Normalized data were tested by a standard paired *t*-test. The *p*-values were then adjusted for a false discovery rate (FDR) (Benjamini and Hochberg 1995). A value of FDR $<$ 0.5 was considered statistically significant. The fold changes of genes were calculated by dividing the normalized signal intensities of genes in compound-treated cells by those in solvent-treated cells. Genes with fold changes $\geq$ 2.0 or $\leq$ −2.0 and FDR values $<$0.5 were selected as differentially expressed genes and further analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (www.genome.ad.jp/kegg/). We used the Web-based gene set analysis toolkit (bioinfo.vanderbilt.edu/webgestalt/) to test the enriched pathways. Microarray data are minimum information about microarray experiments compliant, and raw data have been deposited in the Gene Expression Omnibus (accession number: GSE67504).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was reverse-transcribed for 2 h at 37 °C using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed by mixing cDNA, 2 × Power SYBR Green PCR Master kit and 200 nM of forward and reverse primers. The reaction condition was followed: 10 min at 95 °C; 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates. Relative quantitation (RQ) was calculated using the comparative C_T method ($\Delta\Delta C_T$) which determines the change in expression of a nucleic acid sequence in a test sample (treated group) relative to the same sequence in a calibrator sample (mock group) (Livak and Schmittgen 2001). The ΔC_T value is determined by subtracting the average C_T value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the average C_T value of target gene. The $\Delta\Delta C_T$ value is determined by subtracting the ΔC_T value of mock group from the ΔC_T value of treated group. RQ is calculated as $2^{-\Delta\Delta CT}$. Fold changes were further presented as RQ if the RQ value was $\geq$1, or as −1/RQ if the RQ value is $<$1. The primer ser for each gene was followed: histone