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Hepatoprotective mechanisms of Yan-gan-wan

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Abstract

Background and aims: A herbal prescription, Yan-gan-wan (YGW), has been known to offer hepatoprotective effects in Asian countries for years. This study investigated its mechanisms of action.
Methods: The effects of YGW on CCl_4 induced liver damage were tested in mice and cultured hepatocytes. Microarray analysis screened genes affected by YGW. YGWs effects on the expression of cytochrome P450 (CYP) 2E1 and other isozymes were determined. YGWs effects on TNFα expression and NF-κB activation in Kupffer cells (KC), and TNFα promoter activity in RAW264.7 cells, were also assessed.
Results: Administration of YGW reduced the plasma ALT, centrilobular necrosis, neutrophilic infiltration, and TNFα mRNA in the livers of mice acutely given CCl_4. The in vivo herb treatment reduced ALT release and necrosis of isolated hepatocytes directly exposed to CCl_4. Microarray analysis demonstrated marked reductions in CYP4A10 and 4A14 by YGW but no changes in other CYP isozymes as confirmed by immunoblot analysis. The herb treatment suppressed LPS-stimulated TNFα release in vivo and by cultured KC. Direct addition of the aqueous herb extract suppressed NF-κB activation by KC and TNFα promoter activity in RAW cells under LPS stimulation. This activity to suppress TNFα expression was largely separated into gel filtration fractions with the molecular size of 102–107 Da. YGW also attenuated liver fibrosis induced by chronic treatment of CCl_4 or porcine serum.
Conclusions: The protective effects of YGW on CCl_4 hepatotoxicity are due in part to inhibition of KC NF-κB activation and TNFα expression by small water soluble molecules, and *may also be related to* suppressed hepatic expression of CYP4A10 and 4A14 that are considered as alternative prooxidant cytochromes.

Keywords: Carbon tetrachloride; Liver fibrosis; Kupffer cells; TNFα; CYP4A10; CYP4A14

1. Introduction

Herbal medicine has been used in China for 1000 of years and has recently attracted the interest of modern scientific communities as alternative medicine. However, the efficacy of the herbal medicine is claimed largely based on clinical experiences and its active constituents and fundamental mechanisms have just begun to be investigated. Yan-gan-wan (YGW), an ancient herbal remedy [1] is a mixture of the extracts from eight medicinal herbs and its known ingredients include phenols (ferulic acid, coumaric acid, angelicin, paeonols), polyphenols (tannin, gallic acid), and flavonoids (quercetin, kaempferol). It is known to provide therapeutic and preventive efficacies for liver diseases in Asian nations for many years, and to date no adverse side effects have been reported. More recently, several case studies have been reported to attribute its therapeutic effects on acute hepatitis and chronic active hepatitis to its herbal components such as Chuan-Xiong (Ligusticum chuanxiong Hort), Dong-Quai (Angelica sinensis Diels) and Bai-Shan (Paeonia lactiflora Pall). These effects included improved ALT levels, jaundice,

Abbreviations: YGW, Yan-gan-wan; CYP, cytochrome P450; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; RT-PCR, reverse transcription-polymerase chain reaction; RNA, ribonucleic acid; cDNA, complimentary deoxyribonucleic acid; CCl_4, carbon tetrachloride; ALT, alanine aminotransferase; PPARγ, peroxisome prolierator-activated receptor γ

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recovery rate and liver fibrosis [2 for review]. However, the cellular and molecular mechanisms by which YGW exerts its hepatoprotective effects are largely unknown. The present study was aimed at understanding whether and how YGW prevents acute hepatotoxicity induced by CCl_4 and liver fibrosis induced by repeated injection of CCl_4 or porcine serum. Our results demonstrate that YGW indeed ameliorates the extent of hepatic necrosis and fibrosis induced by CCl_4. They further suggest that the hepatoprotective mechanisms of YGW against acute or chronic CCl_4 injury are not due to alterations in the levels and activities of the enzymes involved in the metabolism of the drug. The mechanisms appear to reside in both parenchymal and Kupffer cells: the former possibly related to selective inhibition of the expression of CYP4A isoforms and the latter involving suppressed TNFα expression.

2. Materials and methods

2.1. In vivo acute CCl_4 toxicity

Animal protocol was approved by IACUC at the University of Southern California according to the NIH guidelines. Male C57BL/6 mice (Charles River Co., Wilmington, MA) were pre-treated with YGW or starch (placebo) via gastric gavage (300 mg/kg, Sheng-Pu Pharmaceuticals, Taipei) and a single dose of CCl_4 (20 μl/kg as 0.2% in mineral oil, Sigma–Aldrich, St. Louis, MO) was injected intraperitoneally. Mice were sacrificed 16 h thereafter and blood was collected for determination of plasma ALT using a kit (Sigma, St. Louis, MO). Livers were fixed in 3% paraformaldehyde for hematoxylin and eosin staining and immunostaining of myeloperoxidase.

2.2. Microarray gene expression analysis

Total RNA was extracted from the livers of mice pretreated with YGW or starch (300 mg/kg/day) [3]. Microarray analysis was performed on the RNA samples using GeneChip® expression analysis by Affymetrix. Changes of gene expression above two-fold were considered as significant.

2.3. TNFα RT-PCR assay

Liver RNA (2 μg) was reverse transcribed into cDNA and the synthesized cDNA was amplified by 30 cycles of PCR using specific primers as described [4,5]. β-actin was used as a house keeping gene for which 25 cycles of amplification were performed.

2.4. In-vitro CCl_4 toxicity in hepatocytes

Hepatocytes were isolated by the Cell Culture Core of the USC Research Center for Liver Diseases by liver perfusion with collagenase [6] from mice pretreated with YGW or starch (300 mg/kg/day for 2 weeks). CCl_4 (66%, v/v in ethanol) was added to hepatocyte cultures (20 μl/ml) [7]. The media were collected for the determination of ALT at 10, 20, and 30 min. In a separate experiment, cultured hepatocytes ($0.5 \times 10^6/27\ cm^2$) were treated with CCl_4 (1 μl/ml) for 1 h, nuclei were stained with Hoechst 33258 (8 μg/ml, Molecular Probes, Eugene, OR) [8] followed by staining of the necrotic nuclei with Sytox green (1 μM, Molecular Probes, Eugene, OR) [9].

2.5. CYP isoform and glutathione assays

Liver microsomes were prepared from the YGW or starch treated mice by differential centrifugation. Proteins (10 μg) were electrophoresed on 9% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were incubated with rabbit anti-rat CYP2E1 (from Dr. B.J. Song, NIAAA), rabbit anti-rat CYP3A, rabbit anti-CYP2B1 (from Dr. J. Halpert, University of Texas Medical Branch at Galveston, TX), rabbit anti pig P450 reductase (from Dr. B.S. Masters, University of Texas Health Science Center, San Antonio, TX) or sheep anti-rat CYP4A (from Dr. G. Gibson, University of Surrey School of Biological Science). Blots were incubated with goat anti-rabbit HRP or rabbit anti-goat HRP secondary antibodies (ICN Biomedicals, Costa Mesa, CA) and analyzed by enhanced chemiluminescence. The testosterone hydroxylation was used to determine the catalytic activity of CYP3A. Testosterone and metabolites were separated and quantified by a Waters HPLC system. The production of 6-hydroxychlorzoxazone was used to determine the catalytic activity of CYP2E1 by HPLC as described by Barmada et al. [10]. CYP2B and CYP1A associated catalytic activities were determined by the dealkylation of pentoxyresorufin and ethoxyresorufin as described by Lubet et al. [11,12] and adapted for microtiter plates as described by Kennedy and Jones [13]. Total glutathione (GSH) content was determined in YGW or starch treated mouse livers without or with CCl_4 treatment by recycling assay as previously described [14].

2.6. Lipopolysaccharide (LPS)-induced TNFα release in vivo and in vitro

Male Wistar rats were gavaged with YGW or starch (300 mg/kg/day) for 2 weeks and LPS was administered (50 μg/kg) (Sigma–Aldrich, St. Louis, MO) via a central venous catheter under general anesthesia. Blood was collected at 0, 0.5, 1, 2, 3 h for TNFα determination by an ELISA kit (R&D System, Minneapolis, MN). Kupffer cells were isolated from normal, YGW or starch treated rats by the Non-Parenchymal Liver Cell Core of the Research Center for Alcoholic Liver and Pancreatic Diseases as published [15]. The cells were treated with LPS (100 or 500 ng/ml) for 4 h for determination of TNFα release. The YGW or starch (placebo) extract was prepared by suspending and mixing in the serum-free media (35 mg/ml), centrifuging at $1500 \times g$ for 15 min, and filter-sterilizing the supernatant. The extract