PXR Mediated Protection against Liver Inflammation by Ginkgolide A in Tetrachloromethane Treated Mice

Nanhui Ye, Hang Wang, Jing Hong, Tao Zhang, Chaotong Lin and Chun Meng*

Institute of Pharmaceutical Biotechnology and Engineering, College of Biological Science and Biotechnology, Fuzhou University, Fuzhou, Fujian, 350108, China

Abstract

The pregnane X receptor (PXR), a liver and intestine specific receptor,, has been reported to be related with the repression of inflammation as well as activation of cytochrome P450 3A (CYP3A) expression. We examined the effect of PXR on tetrachloromethane (CCl4)-induced mouse liver inflammation in this work. Ginkgolide A, one main component of *Ginkgo biloba* extracts (GBE), activated PXR and enhanced PXR expression level, displayed both significant therapeutic effect and preventive effect against CCl4-induced mouse hepatitis. siRNA-mediated decrease of PXR expression significantly reduced the efficacy of GBE, one of most commonly used herbal medicines, has been used clinically for curing peripheral vascular diseases in China, France and Germany. The recent research showed that the GBE complex exhibited hepatoprotective effects against carrageenan-induced acute and chronic liver injuries in rats, but the exact hepatoprotective mechanism of GBE was not clear (Abdel-Salam et al., 2004). Previous rodent studies indicated that continuously feeding GBE influenced pharmacokinetics of other drugs, including shortening barbiturate-induced narcosis (Brochet et al., 1999; Kubota et al., 2003) and reducing the hypotensive action of nicardipine (Kubota et al., 2003).

INTRODUCTION

PXR, known as a xenobiotic sensor, is a nuclear receptor activated by numerous xenobiotic compounds. It was shown to both biochemically and genetically activate expression of CYP3As, CYP2Bs, UGT1A1, ABCB1, and MRP2 to detoxify and clear xenobiotics from the body (Kliewer et al., 1998; Kliewer et al., 2002; Austin et al., 2015). PXR belongs to the nuclear receptor superfamily, members of which are transcription factors characterized by a ligand-binding domain and a DNA-binding domain. Ligand-activated PXR regulates expression of target genes through heterodimerizing with 9-cis retinoic acid receptor alpha (RXRα) to act on promoters.

A few studies have also shown that PXR has potential anti-inflammatory effects. Single-nucleotide polymorphisms linked to a decrease in PXR activity or expression level have resulted in inflammatory bowel disease in patients (Langmann et al., 2004; Dring et al., 2006; Shah et al., 2006; Reyes-Hernandez et al., 2014; Zhang et al., 2014). PXR-null mice were much easier subjected to colitis than wild-type mice (Shah et al., 2006). One possibility of anti-inflammation mechanism is that PXR could afford the intestinal epithelial barrier based on its detoxification properties. The other possibility is involved with cross-talk between PXR and NF-κB signaling pathways (Gu et al., 2006). They reported that p65, one subunit of NF-κB, repressed PXR association with its target genes' promoters through competitively combining RXRα. Inversely PXR activation inhibited the activity of NF-κB in a dependent manner of ligands activation (Zhou et al., 2006).

GBE, one of most commonly used herbal medicines, has been used clinically for curing peripheral vascular diseases in China, France and Germany. The recent research showed that the GBE complex exhibited hepatoprotective effects against carrageenan-induced acute and chronic liver injuries in rats, but the exact hepatoprotective mechanism of GBE was not clear (Abdel-Salam et al., 2004). Previous rodent studies indicated that continuously feeding GBE influenced pharmacokinetics of other drugs, including shortening barbiturate-induced narcosis (Brochet et al., 1999; Kubota et al., 2003) and reducing the hypotensive action of nicardipine (Kubota et al., 2003).
The reason that GBE effecting pharmacodynamics and pharmacokinetics of drugs was related to activity of cytochrome P450 (P450) enzymes. In rat model GBE increased hepatic expression of CYP2B (Shinozuka et al., 2002; Umegaki et al., 2002), one important member of P450 family. It becomes clear that some components in GBE (including flavonoids and terpene triacontanes) are responsible for P450 activities. Moreover some studies on rodent models showed that drug-drug interactions might be mediated by PXR as did in human (Kliewer et al., 2002).

The constitution of GBE used in previous studies was not uniform. For example, levels of the terpene triacontanes in the extracts used in Shinozuka and Umegaki studies (Shinozuka et al., 2002; Umegaki et al., 2002) were greater than those in many of the commercially available GBE which contain only 6% terpene triacontanes (van Beek, 2002). Shinozuka (Shinozuka et al., 2002) reported that two main components in GBE, bilobalide and Ginkgolide A, played different roles in the modulation of CYP2B1 and CYP3A23 gene expression and enzyme activities in rat model. In this work we investigated effect of PXR on hepatitis and colitis in mice for a more thorough understanding of the relationship between PXR and GBE components in anti-inflammation process.

**MATERIALS AND METHODS**

**Materials**

A standardized powder form of Ginkgolide A and flavonoids were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN), Ips, and nicardipine were purchased from Sigma-Aldrich. All other common reagents not listed were purchased from Sigma-Aldrich or other common vendors.

**Animal experiments**

The animal studies were conducted in accordance with the guidelines of the China Council on Animal Care. 4-week-old male C57BL/6 mice (14-16 g) were purchased from the Laboratorial Animal Center of Fujian medical University, China. Mice used in the study were housed 6 per cage and were allowed to acclimate for a period of 3 days prior to the start of the study. Animals were kept in a temperature-controlled facility with 12-h light/dark cycles and were free access to regular rodent chow (commercial rodent diet from the Laboratorial Animal Center of Fujian Medical University) and tap water. In this work we characterized the development of hepatitis and colitis in mice subjected to CCl4 gastric perfusion. For study of effect of Ginkgolide A on micadipine metabolism, mice were divided into 3 groups (untreated control, Ginkgolide A induction and flavonoids induction). Ginkgolide A, flavonoids were orally dosed once daily for 3 consecutive days using corn oil as vehicle respectively. Control group were treated only with corn oil. After treated with nicardipine by gavage, mice were anesthetized with pentobarbital and get blood from eye socket and sacrificed in different time, and the livers were immediately removed for total RNA isolation (Kubota et al., 2003). The concentrations of alamicinotransferase (ALT) and aspartate aminotransferase (AST) in sera were determined by enzymatic assay with R1100TM autochemical analyzer.

**Macroscopic and histologic assessment of hepatitis and colitis**

The livers and colons were examined to evaluate the macroscopic lesions according to the conglutination state among the organs. One part of the liver and colon specimens was fixed overnight in 4% paraformaldehyde and embedded in paraffin. The other parts of the liver and colon were used for mRNA quantification of PXR, TNFα and CYP3A11 (Ameho et al., 1997).

**PXR knocking down mice construction**

The PXR knock down mice were created by transfecting PXR-targeting short hairpin RNAs on plasmid PCI(neo) (Fig. 1). The short hairpin RNA designed for knocking down PXR drove by mouse H1 promoter. AGATCT(BgII) GAAACGCTGACGTCACTAACCCCTCCCAAAGGAATCTCGTACGCTAGCCGGTACCGATGCTAGCCGGGAAACTCCAGCGGCGTGGCGCCCTGGAAGGAGAGTGGCTGGAGGAACGGGAGGTTGGGCCCATGAAATTGGCATGGCTCATTGGTTCCTGGAATACCCATAAACGTGAATTGCTTTGTGAATGTCGAATTGGGAGAAGTCGCTTATCGTATGATGCTAGCAGACCG (H1 promoter) GCTAGC(NheI) CGCGAACAGGCCACGTGCATTTCAGAGAAATAGCAGTGCCCTGTCC TTTTTTGGCC(NOT I) (Hairpin).

Fig. 1. Plasmid construction with a hairpin used for silencing PXR in mice (The hairpin structure DNA was transcribed with H1 promoter). AGATCT(BgII) GAAACGCTGACGTCACTAACCCCTCCCAAAGGAATCTCGTACGCTAGCCGGTACCGATGCTAGCCGGGAAACTCCAGCGGCGTGGCGCCCTGGAAGGAGAGTGGCTGGAGGAACGGGAGGTTGGGCCCATGAAATTGGCATGGCTCATTGGTTCCTGGAATACCCATAAACGTGAATTGCTTTGTGAATGTCGAATTGGGAGAAGTCGCTTATCGTATGATGCTAGCAGACCG (H1 promoter) GCTAGC(NheI) CGCGAACAGGCCACGTGCATTTCAGAGAAATAGCAGTGCCCTGTCC TTTTTTGGCC(NOT I) (Hairpin).
Table 1. PCR primers used in this article

| Name       | Direction | Sequence               | Product size |
|------------|-----------|------------------------|--------------|
| β-actin    | Forward   | GGTCTCAAAACATGATCTGGG  | 238 bp       |
|            | Reverse   | GGGTCAAGAAGGACTCTATG   |              |
| IκBα       | Forward   | CGTCTTTTATGGTAGGATCGAC | 196 bp       |
|            | Reverse   | ACCACTGAGGTCAGTCACCT   |              |
| Promoter 1 | Forward   | AGCCCTATTCTCAAC        | 197 bp       |
| of IκBα    | Reverse   | GCACAAAGAAAGTCGCC      |              |
| Promoter 2 | Forward   | AATGCAGGACCTCAAC       | 215 bp       |
| of IκBα    | Reverse   | ACAGCAGGCTTTATCC       |              |
| PXR        | Reverse   | TCCAGGGGCGACGGTGTA     | 85 bp        |
|            | Reverse   | GCAGGATATGGGGACTACAC   |              |
| RXRα       | Forward   | CTTTGACAGGCTAAGAGGC    | 172 bp       |
|            | Reverse   | ACGCTCTAGTGACGCTACAACC |            |
| TNFα       | Forward   | TCAAGCGAGGACGCAGAA     | 279 bp       |
|            | Reverse   | GCCACAGGACGGAATG       |              |

1:5 (wt/vol). The liposome-siRNA mixture was injected via the mouse tail vein according to the manufacturer’s protocol. The negative control mice were only received blank plasmid PCI mixed with Lipofectamine™ 2000.

**Determination of mRNA expression**

Total RNA was isolated from mouse liver using the Quick-Prep RNA extraction kit (Amersham Biosciences Inc.) according to manufacturer’s protocol. cDNA was synthesized from 0.5 μg of RNA using the first strand cDNA synthesis kit (MBI Fermentas), mRNA levels of various genes were determined by semiquantitative real-time PCR using SYBR Green I according to Abi 7300 protocol description. Primers were synthesized by the DNA Synthesis Centre, Sangong, China. Primer sequences for PCR in this research are listed in Table 1. All mRNA levels were normalized to β-actin mRNA. Normalization to actin mRNA was found to give comparable results.

**EMSA**

Nuclear protein extracts from mouse liver cells treated with CCl4 were prepared for EMSA as described earlier (Gu et al., 2006; Han et al., 2008) with a little change. Nuclear proteins (5 μg) from cells were incubated for 30 min in a reaction mixture containing 40 mM KCl, 1 mM MgCl2, 0.1 mM EGTA, 0.5 mM dithiothreitol, 20 mM Heps, pH 7.9, 4% Ficoll (400 K) and PCR product of IκBα. PCR product 1 contained PXR binding site 1 (-10634−-10621) or product 2 contained site 2 (-3164−-3151). After incubation for 30 min at room temperature, the reaction mixtures were separated by electrophoresis in 4.0% agarose gel. The gel of each lane was cut into 0.5 cm long to extract DNA for analysis of DNA shift with PCR.

**Statistical analysis**

All studies were performed using n=5. All data are expressed as means ± standard deviation (SD). Statistical significance was analyzed by a one-way ANOVA followed by the Newman-Keuls multiple range test (SPSS version 12.0, SPSS, Inc., Chicago, IL, USA). Single (*), double (**) and triple (***) marks represent statistical significance in \( p<0.05 \), \( p<0.01 \) and \( p<0.001 \), respectively.

**RESULTS**

**Therapeutic effect of Ginkgolide A in treating CCl4-induced mice hepatitis**

Gastric perfusion of CCl4 led to the adhesion between the liver and the adjacent tissues mediated by white tissue in the positive model mouse group (Fig. 2A). Whereas control group and high dose Ginkgolide A treated group had no macroscopic lesions in both livers and colons. Hematoxylin-eosin-stained liver tissue sections revealed that CCl4-induced acute inflammation, massive and severe hepatocyte necrosis at the centrilobular zone of mouse livers (Fig. 2B). The results showed that severe hepatotoxicity and colitis were induced by gastric perfusion of CCl4 (5 mL/kg) in corn oil (1:1) at a dose of 750 mg/kg/day for 3 days. We also observed the obvious disruption of the sinusoidal and lobular architecture of the liver in the positive model group. Cells exhibited a regular arrangement surrounding the liver tube in normal mouse livers. Administration of CCl4 induced large areas of liver tissue necrosis and lead to most liver cells in an irregular arrangement. Different dose Ginkgolide A (from 50 to 200 mg/kg/day) significantly decreased CCl4-induced liver lesions and reduced CCl4-induced necroinflammatory response in a dose-dependent manner. There was no obvious necroinflammation in groups treated with high dose Ginkgolide A, and mice liver tissue section slides (paraffin embedded) showed no significant difference between control group and high dose Ginkgolide A treated group.

The levels of ALT and AST, which are mainly distributed in liver cell cytoplasm and mitochondria respectively, were usually very low in sera. When liver cells necrotized, ALT and AST were released from liver cells to sera. So their levels in sera are commonly used as an important indicator of hepatitis. Both AST and ALT in sera of CCl4-treated mice showed liver cells and the mitochondria had been severely damaged (Fig. 3). Compared with CCl4-treated positive model mice, the levels of AST and ALT significantly decreased in sera of ginkgolide A treated mice in a dose-dependent manner. Liver tissue slides observation (Fig. 4A) and changes of AST and ALT in sera (Fig. 4B, C) showed that Ginkgolide A also exhibited satisfied preventive effect against CCl4-induced liver necrosis. Our re-
search showed that Ginkgolide A played an important role in GBE mediated anti-inflammatory reaction.

The results showed that feeding Ginkgolide A increased PXR expression levels with a dose-dependent manner in mouse livers (Fig. 5). In order to examine whether the anti-inflammation of Ginkgolide A was mediated by PXR, we investigated the effect of PXR expression levels on CCl₄-induced hepatocyte necrosis through silencing PXR expression in mouse livers. The expression level of PXR was shown that microRNA silenced PXR gene expression in the mouse liver in vivo using tail vein injection (Fig. 6A). PXR mRNA expression was increased in the livers of Ginkgolide A treated hepatitis mice, but less abundantly in livers of PXR-silenced mice. The previous report showed that PXR null mice would have a more proinflammatory stance in the small intestine. Our results of histological examination demonstrated that silence of PXR resulted in spontaneous inflammation in livers as well as intestine inflammation (Fig. 6B). Feeding Ginkgolide A showed no obvious curative effect on hepatitis in PXR-silenced mice. These results indicated that anti-inflammatory response of Ginkgolide A might be mediated through PXR.

Effects of flavonoids on CYP3A11 activities in mice

We have further investigated whether the anti-inflammatory response of other component in GBE was mediated through PXR. It was reported that flavonoids, another main component in GBE, inhibited human CYP3A4 activity (Ho et al., 2001). We used nicardipine, which is a calcium channel blocker and extensively metabolized by CYP3A type (Shinozuka et al., 2002), as probe drug to determine the effect of flavonoids on the CYP3A11 activities in mice. The metabolism of nicardipine (30 mg/kg, intragastric administration) in controls and drug-treated mice were compared in Fig. 7. The serum concentrations of nicardipine were lower by 43.6% in Ginkgolide A treated group than that in control group. Ginkgolide A accelerated metabolic rate of nicardipine in mice. Oppositely in flavonoids-treated mice the peak plasma concentrations of
nicardipine put off from control’s 10 min to 20 min and the peak concentration increased by 30% than control groups. It indicated that flavonoids inhibited activity of CYP3A11 to lower nicardipine metabolic rate.

Interestingly though flavonoid is an inhibitor of CYP3As, it could increase PXR expression levels (Fig. 8A). If anti-inflammation mechanism of Ginkgolide A was dependent on CYP3A mediated by activation of PXR, flavonoids should have no positive protection results against CCl4-induced hepatocyte necrosis in mice livers. But flavonoids also showed satisfying positive effects against CCl4-induced acute liver inflammation in both therapeutic group mice and preventive group mice. As shown in Fig. 8B, feeding flavonoids for 6 days caused a significant therapeutic effect against the liver lesions and decreased the serum AST and ALT levels with a dose-dependent manner (Fig. 8C). Histologically, livers from flavonoid-diet mice showed obviously therapeutic results compared with control animals. So we can assume that the hepatitis therapies of Ginkgolide A or flavonoids might not be exclusive based on activation of CYP3As in mice, though PXR played an important role in the anti-inflammation process.

**PXR agonist enhances IkBα expression level through activation of PXR**

NF-κB is a major regulator of inflammatory responses stimulated by pro-inflammatory agents, including tumor necrosis factor, viruses, interleukin-1, and bacteria. NF-κB normally resides in the cytoplasm bound by an inhibitory protein known as IkBα. Since NF-κB acts as a mainly inflammation factor, we tested whether activation of PXR affects NF-κB activity. In mouse liver, Ginkgolide A did not inhibit NF-κB expression. But we found that Ginkgolide A enhanced IkBα expression level in a dose-dependent manner as determined through real-time RT-PCR analysis (Fig. 9). In PXR-silenced mice, we also found that IkBα expression level significantly decreased. We have found two PXR/RXRα binding sites in upstream regulation region of IkBα and EMSA showed PXR/RXR could bind these sites dependent on ligand activation (Fig. 10). Based on the above, we believed that activation of PXR inhibited inflammation could be mediated by inhibiting NF-κB activities via enhancing expression of IkBα. High level IkBα interacted
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with NF-κB might inhibit release of NF-κB which decreased the hepatitis and colitis.

**DISCUSSION**

Abdel-Salam et al showed that GBE displayed anti-inflammation efficacy in the acute phase of carrageenan-induced models of acute inflammation in rat (Abdel-Salam et al., 2004). In the present work we have confirmed that both Ginkgolide A and flavonoids in GBE showed obvious anti-inflammatory effect in CCl₄-treated mice. The findings made in this study also showed that: 1) PXR exerts an inhibitory effect on the development of liver inflammations; 2) anti-inflammatory effect of Ginkgolide A or flavonoids might not be mediated through CYP3As. Our findings that anti-inflammatory effect of flavonoids independent on CYP3A11 indicated that PXR exerted suppression efficiency through another pathway independent on CYP3A pathways.

PXR-nude mice tended to develop spontaneous colitis, along with a significant increased level of NF-κB (Shah et al., 2006). NF-κB plays a central role in inflammation through its ability to induce transcription of proinflammatory genes (Baldwin, 1996). Gu et al reported that NF-κB p65 inhibited transactivation of the corresponded genes through directly interacting with the DNA-binding domain of RXRα and preventing PXR/RXRα from binding to the target DNA sequences (Gu et al., 2006). Shah demonstrated that PXR/RXRα complex could not suppress NF-κB activity through direct inhibition of p50/p65 formation inversely (Shah et al., 2006). IκBα could form dimer with the NF-κB to inhibit the NF-κB activities. Then NF-κB-IκB complex primarily located in the cytoplasm and blocked the ability of NF-κB to bind to the target DNA sequences through immigrating in cell nuclear. So IκBα exhibited the anti-inflammatory effect through binding NF-κB (Li and Nabel, 1997). NF-κB activation increases expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 (Chen et al., 1995), so we observed tissue adhesion phenomena in CCl₄-induced inflammation mice in this research. Transcrip-
tional activation of IκBα mediated through Ginkgolide A or flavonoids attenuated liver inflammation and reduced tissue adhesion through inhibiting expression of NF-κB activated proinflammatory factors.

RXRα is a nuclear receptor which exerts its bioactivities by binding, as homodimers or heterodimers with other partners, to specific sequences in the promoters of target genes and regulating their transcription. We have known that RXRα has been shown to interact with CLOCK, TRIM24, nuclear receptor coactivator 2, NPAS2, POU2F1, ITGB3BP, TATA binding protein, IGFBP3, nuclear receptor coactivator 3, NRP1, NCOA6, thyroid hormone receptor beta, retinoic acid receptor alpha, nerve Growth factor IB, TADA3L, BCL3, peroxisome proliferator-activated receptor gamma, PPARG1A, BRD8, liver X receptor beta, MyoD, farnesoid X receptor, calcitriol receptor, about 95 proteins which are involved in biological functions of cell development, differentiation, proliferation, metabolism, and so on (http://www.thebiogrid.org/). Interestingly we found

Fig. 8. Effect of flavonoids on PXR expression in mouse liver and its therapeutic effect on CCl4-induced hepatitis in mice. (A) PXR expression levels in mouse liver treated with vehicle, 100 mg/kg/d ginkgole A and 100 mg/kg/d flavonoids respectively. (B) liver histological sections of mice treated with vehicle, CCl4, and 200 mg/kg/d flavonoids (6 days) after CCl4 treated. (C) Effect of flavonoids on ALT and AST level in sera of CCl4-treated mice. Control mice receiving vehicle only; model mice receiving CCl4 (5 ml/kg/d), 1, 2, and 3 group of mice receiving flavonoids 50, 100, and 200 mg/kg/d respectively 3d before the administration of CCl4. Animals were killed 6 d after flavonoids treatment (The red arrows showed the necrosis of livers).

Fig. 9. Effect of PXR activation on IκBα expression level in mouse liver. Control: Control mouse; 1: mouse treated with 50 mg/kg/d Ginkgolide A for 3d; 2: mouse treated with 100 mg/kg/d Ginkgolide A for 3d; 3: PXR-silencing mouse; 4: PXR-silencing mouse treated with 50 mg/kg/d Ginkgolide A for 3d; 5: PXR-silencing mouse treated with 100 mg/kg/d Ginkgolide A for 3d.
that activation of some other partners of RXRα also exhibited anti-inflammatory activity, such as liver X receptor, farnesoid X receptor, PPAR, RAR (Devchand et al., 1996; Muller and Bendtzen, 1996; Decula and Cantorna, 2001; Desreumaux et al., 2003; Liu et al., 2009). Whether other RXRα partners could regulate NF-κB activities to exert anti-inflammatory effect is a very attracted question. We will investigate the potential anti-inflammatory mechanism of PXR in the future work.

CONFLICT OF INTEREST

No conflict of interest is reported.

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