Ginseng Extracts Facilitate Cytochrome P450 Xenobiotic Metabolism in Primary Cultures of Rat Hepatocytes

Atsushi Kawase,[a] **Fumiaki Takeshita,**[b] **Ayano Yamada,**[a] **Kazuya Murata,**[b] **Hideaki Matsuda,**[b] **Keiichi Samukawa,**[c] **and Masahiro Iwaki*,**[a]

[a]*Department of Pharmacy, Kinki University, 3–4–1 Kowakae, Higashi-Osaka, Osaka 577–8502, Japan,* [b]*Department of Pharmaceutical Sciences, Kinki University, 3–4–1 Kowakae, Higashi-Osaka, Osaka 577–8502, Japan and* [c]*Department of Pharmacology, Osaka City University Medical School, 1–4–3 Asahimachi, Abeno-ku, Osaka 545-0051, Japan*

A 70% methanol extract from red ginseng (steamed and dried roots of *Panax ginseng* C. A. Meyer, a kind of Ginseng Radix) has been shown to have various actions on physiological functions. We investigated whether the ginseng extract (Ext.) affected the mRNA expression of cytochrome P450 (CYP) CYP1A1, 2B1, 2C11, 2D1, 3A1, and 3A2 in rat primary hepatocytes. After treatment with ginseng extract, the levels of CYP3A1 and 1A1 mRNA were significantly increased compared with those of the control. The increased protein levels of CYP3A1 were also observed after treatment with Ext. The mRNA levels of other examined CYPs exhibited little change. The mRNA levels of the pregnane X receptor, but not the constitutive androstane receptor, both transcription factors for CYPs, also significantly increased after treatment with ginseng extract. These results raise the possibility that ginseng Ext. promotes xenobiotic metabolism via an increase in CYP3A1 and 1A1 expression.

Key words —— ginseng, extract, cytochrome, cytochrome P450, rat hepatocyte

INTRODUCTION

Ginseng Radix is an important crude drug that has been used since ancient times to improve constitutional tendencies toward overall poor body condition, to promote appetite, to increase vitality and to reduce over-sensitivity to cold. Pharmacological evidence shows that ginseng improves blood circulation and accelerates both metabolism and digestion. In the Japanese Pharmacopoeia Fifteenth Edition (2006), two kinds of Ginseng Radix, namely, red ginseng [steamed and dried root of *Panax ginseng* (*P. ginseng*)] and white ginseng (peeled and dried root of *P. ginseng*, without steaming) are described. Conclusive evidence indicates that red ginseng is superior to white ginseng as the former contains numerous effective components[1, 2] and possesses stronger physiological activity compared with the latter.[3–5]

Cytochrome P450 (CYP) plays an important role in metabolism of xenobiotics and exogenous molecules, including drugs, environmental chemicals and natural plant products in various tissues such as the liver and small intestine. For example, CYP3A participates in metabolism of about 50% of commercially available drugs. Nuclear receptors such as the pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3) are involved in the regulation of CYP transcription after response to xenobiotics and endogenous toxins.[6–8]

In this study, we conducted *in vitro* experiments using rat hepatocytes to evaluate the effects of a 70% methanol extract (Ext.) from red ginseng on the mRNA levels of CYPs. There are few reports on the effects of Ext. on the mRNA levels of various xenobiotic metabolizing CYPs and the nuclear receptors involved in their regulation. The alterations of CYP expression could also affect the pharmacokinetics of a concomitant drug. We examined CYP1A1, 2B1, 2C11, 2D1, 3A1, and 3A2 mRNA expression because the amounts of these CYPs are relatively high in rat liver. The protein levels of CYP3A1 were also determined. To clarify the effects of Ext. on the nuclear receptors, we also examined the mRNA levels of CAR and PXR.

MATERIALS AND METHODS

Chemicals —— The crushed roots of red ginseng were extracted twice with 70% methanol under reflux, for 2 hr. The Exts. were evaporated under re-

*To whom correspondence should be addressed: Department of Pharmacy, Kinki University, 3–4–1 Kowakae, Higashi-Osaka, Osaka 577–8502, Japan. Tel.: +81-6-6721-2332; Fax: +81-6-6730-1394; E-mail: iwaki@phar.kindai.ac.jp

duced pressure and then lyophilized to give two 70% methanol Ext. (yield: 37–43%). Aprotinin, dexamethasone (DEX), Williams' medium E and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Collagenase (type I) was purchased from Wako Pure Chemicals (Osaka, Japan). Phenobarbital (PB) was obtained from Sankyo (Tokyo, Japan). GlutaMAX^{TM} Supplement I, the primers for polymerase chain reaction, and TRIzol for isolation of total RNA was obtained from Invitrogen (Carlsbad, CA, U.S.A.). Prime Script-RT reagent Kit and $2 \times$ SYBR Premix Ex Taq were purchased from TaKaRa (Shiga, Japan). All other chemicals were reagent grade products obtained commercially.

Animals —— Six- to 7-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in a temperature- and humidity-controlled room and were allowed free access to standard laboratory chow and water. The experiments were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University School of Pharmaceutical Science.

Isolation, Culture, and Treatment of Rat Hepatocytes —— Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Hepatocytes were isolated by a *in situ* two-step collagenase perfusion of rat livers (200–250 g) according to the method of Seglen,[9] and cell viability was determined by trypan blue exclusion. Cells were seeded at a density of 1×10^5 cells/cm^2 on a collagen coated six-well plate (Sumitomo Bakelite, Tokyo, Japan) in Williams' medium E supplemented with 5% fetal bovine serum, 1 nM insulin, 1 nM dexamethasone, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM GlutaMAX^{TM} Supplement I and were incubated at 37°C in the presence of 5% CO$_2$ and 95% air. After an initial attachment period of 3 hr, the medium was replaced with fresh Williams' medium E with the addition of the whole supplements and 5 U/ml aprotinin except serum (dubbed serum-free medium). At 24 hr after plating, the medium was replaced with fresh serum-free medium and the cultured hepatocytes were treated for 24 hr with Ext. (100–1000 µg/ml) in phosphate buffered saline (PBS) (pH 7.4) or PBS alone (control). In the positive control experiments, cultured hepatocytes were treated with PB (CAR ligand) (100 µM) or DEX (PXR ligand) (10 mM) in dimethyl sulfoxide (DMSO). The final DMSO concentration in the culture medium was 1.0% (v/v).

Isolation of Total RNA, Reverse Transcription and Real-Time PCR Analysis —— Total RNA was extracted from the treated hepatocytes using TRIzol according to the manufacturer's instructions. The reverse transcription was performed for 15 min at 37°C and 5 s at 85°C using a Prime Script-RT reagent Kit. The reverse-transcribed cDNA was used as template for real-time RT-PCR. Amplification was performed in a 50 µl reaction mixture containing $2 \times$ SYBR Premix Ex Taq and 0.2 µM of a primer set of 18S ribosomal RNA (18S rRNA) as an endogenous reference. For all PCR amplifications, we used oligonucleotide sequences designed by Primer Express 2.0 (Applied Biosystems, Foster City, CA, U.S.A.), as shown in Table 1. Amplification and detection were performed with an ABI PRISM 7000 (Applied Biosystems). The samples were incubated at 95°C for 10 s, and then subjected to 40 three-step cycles of 95°C for 5 s, 55°C for 20 s, and 72°C for 31 s. In Table 1, the oligonucleotide sequences for each target gene are shown. Data were analyzed using the ABI Prism 7000 SDS Software (Applied Biosystems), particularly using the multiplex comparative method. The relative amounts of the target mRNAs in liver and intestine samples were compared by measuring their Cycle thresholds (C_t). To determine the quantities of the target gene-specific transcripts present in the liver and small intestines, their respective C_t values were first normalized by subtracting the 18S rRNA control C_t values from the test C_t values ($\Delta C_t = C_{t,target} - C_{t,control}$). The concentrations of gene-specific mRNAs in treated hepatocytes relative to those in control cells were calculated by subtracting the normalized C_t values obtained for each treated cell ($\Delta\Delta C_t = \Delta C_{t,Ext.} - \Delta C_{t,Control}$) and the relative concentrations were determined ($2^{-\Delta\Delta C_t}$).

Preparation of Microsomes and Western Immunoblotting —— At the end of the culture period, hepatocyte cultures were rinsed twice with ice-cold PBS. An aliquot (0.5 ml) of ice-cold homogenizing buffer (50 mM Tris-HCl, 150 mM KCl, 2.0 mM EDTA) was added to each well. Cells were scraped from the wells with a rubber policeman, collected, and homogenized by sonication at approximately 40 W for 15 sec. Cell lysates were centrifuged at 9000 g for 20 min at 4°C. Supernatant fractions were collected and centrifuged at 105000 g for 60 min at 4°C. The microsomal pellets were suspended in 250 mM sucrose (0.1 ml), and protein concentrations were measured using the BCA Protein Assay Kit (Pierce Biotechnology, Inc.,

Table 1. Sequences of Primers Specific to Rat CYP Isoforms and Nuclear Receptors Selected from a Unique Region of the Respective Complete cDNA Sequence

CYP3A1 NM_173144
    forward: 5′-GCCTTTTTTTGGCACTGTGCT-3′
    reverse: 5′-GCATTTGACCATCAAACAACCC-3′

CYP1A1 NM_012540
    forward: 5′- GCTGAGGCTCAACTGTCTTCCA-3′
    reverse: 5′-GGCTTTGCAAGGACAAGGAGA-3′

CYP2B1 J_00719
    forward: 5′-CAAGGAGAGTGGCATTGGAAAA-3′
    reverse: 5′-AGGCCATTCCCAACAGAGAACTG-3′

CYP2C11 NM_019184
    forward: 5′-CGCACGGAGCTGTTTTTGTT-3′
    reverse: 5′-GCAAATGGCCAAATCCACTG-3′

CYP2D1 NM_153313
    forward: 5′-ACGCATCACGAGTTGTGACATT-3′
    reverse: 5′-AGACGGTCTCATCCTTCAGCAC-3′

CYP3A2 NM_153312
    forward: 5′-TTTGCCATCATGGACACAGAGA-3′
    reverse: 5′-GCTTTCCCCATAATCCCCACT-3′

PXR NM_052980
    forward: 5′-GAC GGC AGC ATC TGG AAC TAC-3′
    reverse: 5′-TGA TGA CGC CCT TGA ACA TG-3′

CAR NM_022941
    forward: 5′-CCA CGG GCT ATC ATT TCC AT-3′
    reverse: 5′-CCC AGC AAA CGG ACA GAT G-3′

Ribosomal 18S RNA NM_213557
    forward: 5′-CGC CGC T AGG TGA AAT TC-3′
    reverse: 5′-CCA GTC GGC ATC GTT TAT GG-3′

Rockford, IL, U.S.A.). SDS-polyacrylamide gel electrophoresis was performed using 10% e-Pagell (Atto, Tokyo, Japan) and 10 μg of microsomal protein per well, and resolved proteins were transferred onto Hybond-P PVDF membrane (GE Healthcare, NJ, U.S.A.). Immunoreactive CYP3A1 proteins were detected using a monoclonal antibody against rat CYP3A1 (Oxford Biomedical Research, Oxford, U.K.) and the ECL Plus Western Blotting Detection system (GE Healthcare).

Data Analysis —— Numerical values were expressed as means ± S.D. and statistical analyses were performed using Dunnett's test after analysis of variance (ANOVA).

RESULTS AND DISCUSSION

We investigated whether Ext. affected CYP3A1 mRNA and protein expression in rat hepatocytes (Fig. 1). In the group treated with various concentrations of Ext., the mRNA levels of CYP3A1 were significantly increased compared with that in the control in a dose-dependent manner (Fig. 1a). The protein levels of CYP3A1 were also significantly increased compared that in the control in a dose-dependent manner (Fig. 1b). Rat CYP3A1 is the main CYP3A form in the rat liver and is orthologous to human CYP3A4. The increased levels of CYP3A1 mRNA and protein could affect the metabolism activities for CYP3A1 substrates. To clarify whether the effects of Ext. on CYP were specific for CYP3A1, we determined the mRNA levels of other CYP isoforms after treatment with 500 μg/ml of Ext. In Fig. 2, the mRNA levels of CYP1A1, 2B1, 2C11, 2D1, and 3A2 with or without treatment were shown. The treatment of Ext. caused a significant increase in the mRNA levels of CYP1A1. All other examined CYP isoforms showed little alteration in their mRNA levels after treatment with Ext. However, these results should be interpreted with caution because the level of mRNA expression does not always directly correlate with protein expression and metabolic activity. These results suggested that the metabolic rate of CYP3A1 and 1A1 could be changed by treatment with Ext. Furthermore, the kinetics of the turnover of substrates could be affected by treatment of Ext. *in vivo*. In a previous study, the administration of a single oral dose (10, 20, or 30 mg/kg) of a crude *P. ginseng* extract to adult male rats resulted in a significant increase in hepatic CYP-mediated aminopyrine *N*-demethylation activity.[10] The results shown in Figs. 1 and 2 suggested that Ext. stimulated the common transcription pathway of CYP3A1 and 1A1.

Nuclear receptors are a factor in the regulation of CYP transcription. We examined the effect of Ext. on PXR and CAR mRNA levels. The treatment of the rat hepatocyte with Ext. promoted the mRNA levels of PXR, but not those of CAR. PXR participates in the regulation of both CYP3A and 1A.[11–14] Thus the results of Figs. 1 and 2 raise the possibility that the increase in the PXR mRNA was involved in the up-regulation of CYP3A1 and 1A1 mRNA. PB and DEX both exhibited positive regulation for the examined CYP isoforms regulated by CAR and PXR, respectively (data not shown).

Red ginseng contains many types of ginseng saponins (ginsenosides) such as ginsenoside-Ro, Rg1, Rb1, and Rg3. However, the effects of individual ginsenosides on xenobiotic-metabolizing CYPs are unclear. Further studies are needed to clarify which components increase the mRNA levels of CYP3A1 and 1A1.
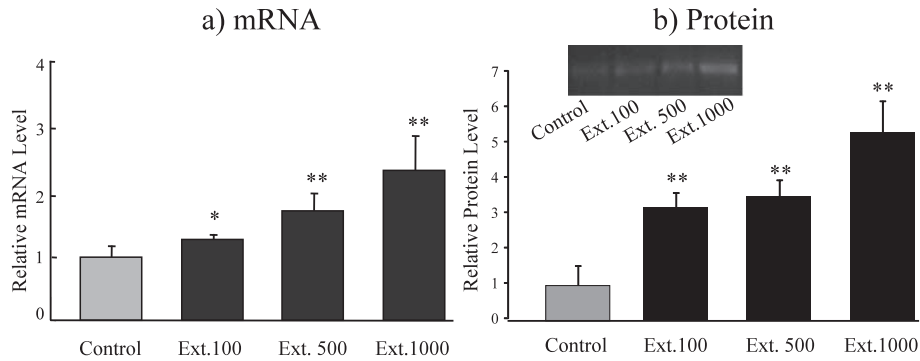
a) mRNA

b) Protein

Fig. 1. Results from the Treatment of 100, 500 and 1000 μg/ml Ext. on CYP3A1 mRNA (a) and Protein (b) Levels in Rat Primary Hepatocytes

Each value represents the mean ± S.D. of four wells. $^*p < 0.05$, $^{**}p < 0.01$, indicates a significant difference from the control.
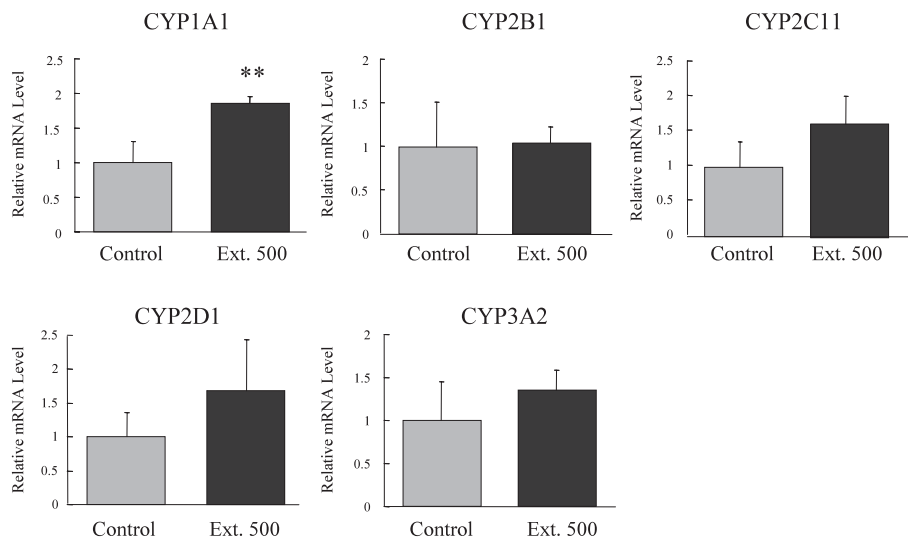
CYP1A1

CYP2B1

CYP2C11

CYP2D1

CYP3A2

Fig. 2. Results from the Treatment of 500 μg/ml Ext. on CYP1A1, 2B1, 2C11, 2D1, and 3A2 mRNA Levels in Rat Primary Hepatocytes

Each value represents the mean ± S.D. of four wells. $^{**}p < 0.01$, indicates a significant difference from the control.
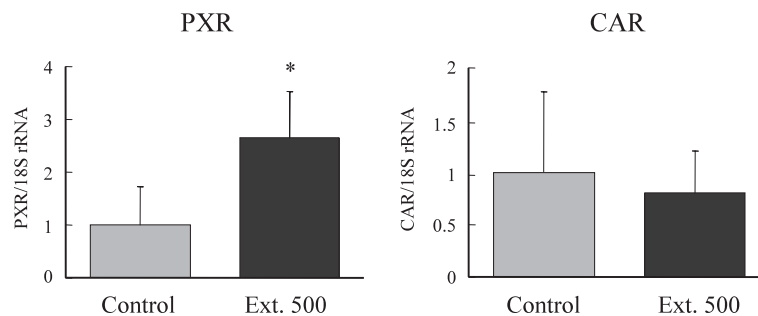
PXR

CAR

Fig. 3. Results from the Treatment of 500 μg/ml Ext. on PXR and CAR mRNA Levels in Rat Primary Hepatocytes

Each value represents the mean ± S.D. of four wells. $^*p < 0.05$, indicates a significant difference from the control.

In conclusion, this study showed that Ext. induced the expression of CYP3A1 and CYP1A1 mRNA following an increase in the mRNA levels of PXR in rat hepatocytes. The significant increased levels of CYP3A1 protein were also observed in treatment with Ext. It is possible that Ext. would have similar effects on xenobiotic metabolism *in vivo*.

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