Strong synergistic induction of CYP1A1 expression by andrographolide plus typical CYP1A inducers in mouse hepatocytes

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Received 22 May 2007; revised 24 June 2007; accepted 11 July 2007
Available online 28 July 2007

Abstract

The effects of andrographolide, the major diterpenoid constituent of Andrographis paniculata, on the expression of cytochrome P450 superfamily 1 members, including CYP1A1, CYP1A2, and CYP1B1, as well as on aryl hydrocarbon receptor (AhR) expression in primary cultures of mouse hepatocytes were investigated in comparison with the effects of typical CYP1A inducers, including benz[a]anthracene, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Andrographolide significantly induced the expression of CYP1A1 and CYP1A2 mRNAs in a concentration-dependent manner, as did the typical CYP1A inducers, but did not induce that of CYP1B1 or AhR. Interestingly, andrographolide plus the typical CYP1A inducers synergistically induced CYP1A1 expression, and the synergism was blocked by an AhR antagonist, resveratrol. The CYP1A1 enzyme activity showed a similar pattern of induction. This is the first report that shows that andrographolide has a potency to induce CYP1A1 enzyme and indicates that andrographolide could be a very useful compound for investigating the regulatory mechanism of the CYP1A1 induction pathway. In addition, our findings suggest preparing advice for rational administration of A. paniculata, according to its ability to induce CYP1A1 expression.

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Keywords: Andrographolide; CYP1A1; CYP1A2; CYP1B1; AhR; Mouse hepatocyte

Introduction

Cytochrome P450s (P450) constitute a superfamily of heme-proteins that play an important role in the metabolism of xenobiotics, including drugs, toxins, and chemical carcinogens (Güengerich, 2000; Güengerich and Shimada, 1998). Of these, P450s, CYP1A1 and CYP1A2 have been shown to be the major enzymes in the metabolism of potential procarcinogens such as polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs, and aryl and heterocyclic amines. In addition to being substrates, PAHs are also inducers of CYP1A1 and CYP1A2 genes. The mechanisms of transcriptional regulation of the two genes are not the same. CYP1A1 is expressed constitutively in several extrahepatic tissues, but not in the liver. However, while CYP1A1 expression has been demonstrated in the liver after inducer treatment, CYP1A2 is constitutively and inducibly expressed only in the liver (Kimura et al., 1986; Iwanari et al., 2002). Aryl hydrocarbon receptor (AhR) has been shown to play central roles in the regulation and induction of CYP1A1 and CYP1A2 by a prototype inducer, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Whitlock, 1999). In addition to the two members of the CYP1A subfamily, CYP1B1, a relatively new member of the superfamily 1 (Brake et al., 1999; Ryu and Hodgson, 1999; Savas et al., 1994), has been postulated to be involved in the metabolism of PAHs such as TCDD through...
AhR and the AhR nuclear translocator (ARNT)-mediated pathway (Ryu and Hodgson, 1999; Savas et al., 1994). Constitutive expression of CYP1B1 was detected in steroidogenic tissues such as adrenal glands, ovaries, and testes, but it was not detected in xenobiotic-metabolizing organs such as liver, kidney, and lung (Iwanari et al., 2002; Savas et al., 1994). Since CYP1 is responsible for activating carcinogenic aromatic amines and heterocyclic amines, to which we are exposed to every day via smoking, diet, and the environment, its regulation is of clear interest.

Andrographolide (3-[2-decachydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-naphthyl]ethyldiene)dihydro-4-hydroxy-2(3H)-furanone) is the major diterpenoid constituent of the plant Andrographis paniculata Nees (Family Acanthaceae), which has been traditionally employed for centuries in Asia and Europe as a folk remedy for a wide spectrum of ailments or a herbal supplement for health promotion, and is nowadays incorporated in a number of herbal medicinal preparations. It is found in the Indian Pharmaco-poeias and is a prominent component in at least 26 Ayurvedic formulas (Madav et al., 1995). In traditional Chinese medicine, it is an important “cold property” herb used to rid the body of heat, as in fevers, and to dispel toxins from the body (Deng, 1978). In Scandinavian countries, it is commonly used to prevent and treat the common cold (Caceres et al., 1997). A. paniculata is one of the top 10 herbal medicines which the Thai FDA has promoted as an alternative medicinal therapy for fever and inflammation. Extensive research has revealed that the whole-plant extract is useful for anti-inflammatory (Shen et al., 2002), antiviral (Calabrese et al., 2000), anticancer (Kumar et al., 2004), and immunostimulatory (Puri et al., 1993; Iruretagoneya et al., 2005) treatments. On the other hand, male reproductive toxicity (Akbarsha and Murugaian, 2000) and cytotoxicity (Nanduri et al., 2004) of this plant have been reported as well. Andrographolide has been reported to show hepatoprotective activity in mice against carbon tetrachloride and paracetamol intoxication (Handa and Sharma, 1990a; Handa and Sharma, 1990b), and to possess several pharmacological activities, including inhibition of iNOS expression (Chiou et al., 2000, 1998), Mac-1 expression, and ROS production (Shen et al., 2002, 2000), and a protective effect against cytotoxicity (Kapil et al., 1993). This compound has recently been shown to work as an anti-inflammatory agent by reducing the generation of ROS in human neutrophils (Shen et al., 2002), as well as preventing microglia activation (Wang et al., 2004) and interfering with T cell activation (Iruretagoneya et al., 2005). Recently, we reported that a crude extract of A. paniculata might induce mouse hepatic cytochrome P450 isoforms CYP1A1 and CYP2B1 via significant increases in ethoxyresorufin O-dealkylase (EROD) and pentoxyresorufin O-dealkylase activities (Jurakamjorn et al., 2006). However, there have been no studies on the impact of andrographolide on the expression of hepatic P450 enzymes. Therefore, it was of interest to examine how andrographolide influences the expression of hepatic P450s. The results of such an inquiry might provide invaluable guidelines for the rational administration and precautions for the use of the herbal plant.

In the present study, the ability of andrographolide to elevate the hepatic expression of CYP1A1, CYP1A2, CYP1B1, and AhR was examined in mouse hepatocytes and compared with that of typical CYP1A inducers, including benzo[a]anthracene (B[a]A), β-naphthoflavone (β-NF), and TCDD. Subsequently, the synergistic effects of concomitant treatment with andrographolide and the typical CYP1A inducers on the induction of CYP1A1 mRNA, protein, and enzyme activity were examined. The findings revealed that andrographolide has a potency to induce CYP1A1 enzyme.

Materials and methods

Materials. Materials for culturing hepatocytes were purchased from Gibco® Invitrogen Cell Culture (Carlsbad, CA), BioWhittaker™ Cambrex Bio-Sciences (Walkerville, MD), and Wako Pure Chemical (Osaka, Japan). Percoll and collagenase (Type I) were products of GE Healthcare Bio-Sciences AB (Uppsala) and the Sigma Chemical Co. (St. Louis, MO), respectively. Andrographolide, resveratrol, and the LDH-cytotoxic test were supplied by Wako Pure Chemical. B [a]A, β-NF, TCDD, ethoxyresorufin, and resorufin were obtained from Sigma Chemical Co. The TaKaRa RT-PCR kit (Perfect Real Time) and SYBR® Green Premix Ex Taq™ (Perfect Real Time) were products of TaKaRa Biomedicals Inc. (Shiga, Japan). The TaqMan® Gene Expression Assays were products of Applied Biosystems (Branchburg, NJ). The antibody against rat CYP1A1 was a generous gift from Dr. Y. Funae (Osaka City University, Osaka, Japan). The Amersham Pharmacia Biotech Co. supplied Hybond-C membranes for blotting. The Dual-Luciferase Reporter Assay System was a product of Promega® (Madison, WI). All other laboratory chemicals were of the highest available purity from commercial suppliers.

Preparation of primary hepatocyte cultures. The liver of a ddY male mouse (Sankyo Laboratories, Shizuoka, Japan) at 8 weeks of age was perfused with collagenase, and viable hepatocytes were isolated by means of Percoll isodensity centrifugation as described (Nemoto and Sakurai, 1995). Standard culture conditions were used as follows: the cells were dispersed in Waymouth MB 752/1 medium containing bovine serum albumin (2 g/l), insulin (0.5 mg/l), transferrin (0.5 mg/l), and selenium (0.5 µg/l), and seeded in dishes at a density of 5 × 10⁵ cells/10 ml/100-mm collagen-coated dish. The Waymouth medium did not contain phenol red, a pH indicator, to exclude the possibility of estrogen-like action. Depending on the presence of cell attachment factors, the hepatocytes anchor to the dishes within 3 h and subsequently form a monolayer. The culture dishes were maintained at 37 °C in a CO₂-humidified incubator. The medium was renewed 3 h after plating and then, after 24 h, the treatment with andrographolide and/or typical CYP1A inducers was performed immediately following the medium change, unless otherwise indicated. The inducers were dissolved in DMSO, which itself had no influence on enzyme activity at the routinely employed maximum concentration of 0.1% (Nemoto and Sakurai, 1992). The cells were harvested after another 24 h to prepare total RNA or microsomal proteins as described elsewhere (Jurakamjorn et al., 1998). These culture conditions were convenient for maintaining CYP1A1 and CYP1A2 expression (Nemoto and Sakurai, 1992) and the concentrations of added compounds were proved to be non-cytotoxic by the methods of the LDH-cytotoxic test and the normalized level of GAPDH.

Real-time RT-PCR. Mouse CYP1A1, CYP1A2, CYP1B1, AhR, and GAPDH mRNAs were quantified by real-time RT-PCR. Hepatic total RNA was reverse-transcribed and cDNA was amplified under the conditions recommended by the supplier (TaKaRa Biomedicals Inc., Shiga, Japan) of the TaKaRa® RT-PCR kit (Perfect Real Time) using specific TaqMan® Gene Expression Assays (Invento- ried) for Cyp1a1 (assay ID, Mm00487218_m1), Cyp1a2 (Mm00487224_m1), Cyp1b1 (Mm00487229_m1), and AhR (Mm00478932_m1), as well as the SYBR® Premix Ex Taq™ (Perfect Real Time) for GAPDH, in which the forward and reverse primers were 5′-TCATCACGGCAAATGACG-3′ and 5′-TAGACTCCAGCATACTCAGC-3′, respectively. The specificity of amplification of GAPDH cDNA was confirmed by both polyacrylamide gel electrophoresis and the dissociation curve of the product. Real-time PCR was
performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Branchburg, NJ) with ABI Prism® 7000 SDS software. The conditions of each PCR cycle were as follows: denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 1 min. The amplified products of CYP1A1, CYP1A2, CYP1B1, and AhR cDNAs were detected directly by monitoring the fluorescence of the reporter dye (FAM), for which an increase in fluorescence signal was detected only if the target sequence was complementary to the probe and amplified by the PCR. The amplified PCR products of GAPDH were monitored directly by measuring the increase in the signal of SYBR® Green that was bound to double-stranded DNA amplified by PCR.

**Western blotting of microsomal proteins.** The microsomal fraction was prepared by ultracentrifugation of the 10,000×g supernatant of monolayer-cultured hepatocytes at 104,000×g for 60 min at 4 °C (Nemoto et al., 1989). The microsomal protein concentration was determined as described using bovine serum albumin as a standard (Nemoto and Sakurai, 1992). Sixty micrograms of microsomal protein was resolved by 10% SDS-PAGE and then transferred to a Hybond-C membrane. The P450 species were detected using a rabbit polyclonal antibody against rat CYP1A1 protein that cross-reacted with CYP1A2, followed by a biotinylated goat anti-rabbit IgG and biotinylated horseradish H-avidin complex, and then visualized with 3,3′-diaminobenzidine and hydrogen peroxide.

**Assessment of enzyme activity.** EROD activity was determined by the method of Sakuma et al. (1999) with some modifications. Briefly, monolayer-cultured hepatocytes that have been cultivated for 2 days in a 60-mm collagen-coated dish were incubated with 10 μM ethoxyresorufin, and then a 0.4-ml aliquot of the medium was sampled at specified times. Subsequently, the formation of resoruﬁn was immediately analyzed by spectrofluorometry with excitation wavelength of 530 nm and emission wavelength of 585 nm.

**Results**

Different effects on expression of CYP1A1, CYP1A2, CYP1B1, and AhR mRNAs by andrographolide

The effects of andrographolide on the expression of CYP1A subfamily members and the mediating transcription factor were examined in monolayer-cultured hepatocytes. Andrographolide markedly induced the expression of CYP1A1 and CYP1A2 mRNAs in a concentration-dependent manner (Fig. 1A). Maximal induction of CYP1A1 expression was observed 1 day after the treatment, while that of CYP1A2 was observed later. B[a]A significantly increased the expression of CYP1A1, CYP1A2, and AhR mRNAs in monolayer-cultured hepatocytes. Hepatocytes were isolated for plating in 35-mm collagen-coated dish at the same density as mentioned above. The hepatocytes were transfected with 1.8 μg of the reporter construct containing 3 tandem repeats of Dioxin Responsive Element (DRE) (Yao and Denison, 1992) in pGL3-promoter vector and 0.2 μg of pRL-SV40 vector (as an internal control for transfection) by using Trans Pass D1 Transfection Reagent (New England Biolabs, Ipswich, MA). Three hours after the transfection, the medium was renewed and the treatment with andrographolide and/or TCDD was started. The cells were harvested after incubation for further 24 h. Luciferase activity was measured by the supplier’s recommendation.

**Transient transfection and luciferase assay.** One day before transfection, hepatocytes were isolated for plating in 35-mm collagen-coated dish at the same density as mentioned above. The hepatocytes were transfected with 1.8 μg of the reporter construct containing 3 tandem repeats of Dioxin Responsive Element (DRE) (Yao and Denison, 1992) in pGL3-promoter vector and 0.2 μg of pRL-SV40 vector (as an internal control for transfection) by using Trans Pass D1 Transfection Reagent (New England Biolabs, Ipswich, MA). Three hours after the transfection, the medium was renewed and the treatment with andrographolide and/or TCDD was started. The cells were harvested after incubation for further 24 h. Luciferase activity was measured by the supplier’s recommendation.

![Fig. 1. Modulation of B[a]A-inducible expression of CYP1A1, CYP1A2, CYP1B1, and AhR mRNAs by andrographolide in monolayer-cultured hepatocytes.](image-url)
CYP1A2, and CYP1B1 mRNAs (Fig. 1B), in accord with previous reports (Guengerich and Shimada, 1998; Iwanari et al., 2002; Whitlock, 1999). Interestingly, co-treatment with andrographolide and B[a]A synergistically enhanced the expression of CYP1A1 mRNA, while no such synergistic effect was observed on the expression of CYP1A2. On the other hand, andrographolide could not modify the expression levels of AhR or B[a]A-induced CYP1B1 mRNAs. These observations suggest for the first time that andrographolide, a major diterpenoid lactone in the extract of *A. paniculata*, has a potency to induce CYP1A subfamily.

**Impact of resveratrol on the synergistic modification of B[a]A-inducible expression of CYP1A1 mRNA by andrographolide**

To examine whether AhR influences the regulation of andrographolide-inducible CYP1A1 mRNA expression, resveratrol, an AhR antagonist (Chun et al., 1999), was employed under the same condition in which andrographolide enhanced the induction of CYP1A1 mRNA expression by an AhR ligand, B[a]A. Resveratrol blocked the synergistic action of the combined treatment of andrographolide and B[a]A, whereas the effect of single treatment with either andrographolide or B[a]A was not affected (Fig. 2). These results revealed that AhR participates in the synergistic effect of concomitant treatment with andrographolide and B[a]A on CYP1A1 mRNA expression, at least in part.

**Activation of CYP1A1 mRNA and protein expression by andrographolide and typical CYP1A inducers**

To evaluate the effects of andrographolide on the induction of CYP1A1 mRNA expression by typical CYP1A inducers, the relative mRNA expression of CYP1A1 compared to GAPDH in monolayer-cultured hepatocytes incubated with andrographolide and/or typical CYP1A inducers, including β-NF, TCDD, and B[a]A, was analyzed. Andrographolide significantly induced the expression of CYP1A1 mRNA (62-fold), although the level of induction was relatively low compared to that obtained using typical CYP1A inducers (Fig. 3A). Panel A shows synergistic induction of CYP1A1 expression by co-treatment of typical inducers and andrographolide. Numbers in the figure indicate expression compared with that of the control group (=1), unless otherwise indicated. Concentrations of typical CYP1A inducers were 13 μM for β-NF and B[a]A, and 30 nM for TCDD. NT, non-treatment. Panel B shows the concentration-dependent induction of β-NF-induced CYP1A1 expression by andrographolide. The concentrations of β-NF were 1, 3, 6, or 13 μM.

As shown in Fig. 4, robust induction of CYP1A1 proteins was observed in microsomes from hepatocytes treated with either andrographolide plus β-NF, TCDD, or andrographolide...
plus TCDD. Under the conditions used, it was hardly possible to detect the bands of CYP1A1 protein in extracts from cells treated with either andrographolide or andrographolide plus B[a]A in the figure. The induction and synergism of the induction of CYP1A1 protein expression by different treatments were in accord with the effects on CYP1A1 mRNA expression (Fig. 3A) when the analyses were performed using the optimal conditions for detection of these expression levels.

The activity of EROD, which was reported to be selective for the CYP1A1 enzyme in the liver of mice (Burke et al., 1994), was assessed to confirm the synergistic regulation of the expression of the CYP1A1 enzyme by andrographolide. In accord with the expression of CYP1A1 mRNA and protein, the EROD activity in the cells co-treated β-NF or TCDD plus andrographolide was significantly higher than that in the cells with single treatment with andrographolide, while that in the cells treated concomitantly with B[a]A and andrographolide was almost the same as that in cells treated with either agent alone (Fig. 5).

To examine the possibility of whether andrographolide induced CYP1A1 after AhR activation, the effect of andrographolide on DRE-mediated transcriptional activity was investigated by a luciferase reporter assay. When TCDD was exposed to the cells transfected with the reporter construct containing the 3×(DRE), the luciferase activity was elevated to a level of 9-fold higher than that of control cells transfected with empty vector, and was not enhanced by co-treatment with andrographolide (Fig. 6).

Discussion

The present investigations employing primary mouse hepatocytes as monolayer cultures revealed that andrographolide has a novel potency to induce the CYP1A1 subfamily. In addition, a robust increased expression of UGT1A6 mRNA, which belongs to a battery of AhR-mediated genes, by andrographolide was noted (our unpublished observations). Furthermore, a strong synergistic expression of CYP1A1 mRNA was observed in the presence of andrographolide after treatment with typical CYP1A1 inducers.

The observations that the synergistic induction of CYP1A1 expression by andrographolide was blocked by resveratrol suggests a possibility that AhR-mediated transcription activation pathway could be involved in the mechanism of induction of CYP1A1 mRNA by andrographolide. Furthermore, the increase of luciferase activity seen in TCDD-treated cells transfected with the 3×(DRE), with no enhancement by co-treatment with andrographolide, supported this possibility. Therefore, andrographolide might influence the expression mechanism of CYP1A1 by enhanced efficiency of mRNA processing or inhibition of mRNA turnover.

However, resveratrol has several pharmacological actions, which suggests that the target point of the compound is involved in various signal pathways (Chun et al., 1999; Park et al., 2007; Le Corre et al., 2006); further investigations not restricted to the AhR pathway to reveal the mechanism of the synergism might be required. Furthermore, the mechanisms of transcriptional regulation of CYP1A1, CYP1A2, and CYP1B1 are not the same (Guengerich and Shimada, 1998; Iwanari et al., 2002),
which may have led to our observations that the andrographolide-inducibility of the expression of those isoenzymes differed: andrographolide extensively induced CYP1A1 expression, while it induced CYP1A2 less markedly, and did not induce CYP1B1 expression (Fig. 1).

The metabolism of a drug can be altered by another drug or a foreign chemical, and such interactions can often be clinically significant. P450 enzymes are involved in the metabolism of a plethora of xenobiotics and have been shown to be involved in numerous interactions between drugs and food, herbs, or other drugs (Delgoda and Westlake, 2004). Phytochemical-mediated modulation of P450 activities has been widely studied. For example, some kind of P450 species was reported to be altered by treatment with St. John’s wort and a constituent that example, some kind of P450 species was reported to be altered by treatment with St. John’s wort and a constituent that, for instance, some kind of P450 species was reported to be altered by treatment with St. John’s wort and a constituent that may have led to our observations that the andrographolide-inducibility of the expression of those isoenzymes differed: andrographolide extensively induced CYP1A1 expression, while it induced CYP1A2 less markedly, and did not induce CYP1B1 expression (Fig. 1).

The relevance of andrographolide utilized, A. paniculata, as a medicinal herb was evident. Therefore, some risks associated with the use of this compound might be of interest and further evaluation of andrographolide analogs should be performed. Our findings suggest some possible advice for the rational administration and precautions for using the herbal medicine A. paniculata.

Acknowledgments

This work was partly supported by the JSPS-NRCT Core University Program on Natural Medicine in Pharmaceutical Sciences, Grants-in-Aid from the Japanese Ministry of Education, Culture, Sport, and Science, the Smoking Research Foundation, the Tokyo Biochemical Research Foundation (TBRF), Japan, as well as the Thai Research Fund (TRF), Thailand.

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