Short Communication

Time-Dependent Induction of Hepatic Cytochrome P450 Enzyme Activity and mRNA Expression by Bilobalide in Rats

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Abstract. A single dose by gavage of bilobalide (30 mg/kg) was found to produce a time-dependent induction of hepatic cytochrome P450 (CYP) enzyme activity and protein expression in rats. An RT-PCR study further showed that mRNA expression of CYP2B was maximal at 6 h. Plasma and liver bilobalide concentration in rats following administration of Ginkgo biloba extract equivalent to bilobalide of approximately 40 mg/kg showed a similar response to that exhibited by mRNA expression. These findings suggest that bilobalide markedly induced hepatic CYPs, but the induction could be mitigated due to rapid elimination from the liver.

Keywords: bilobalide, cytochrome P450 (CYP) induction, herb–drug interaction

Ginkgo biloba extract (GBE) is one of the most popular medicinal plants widely used for the treatment of dementia, depression, dizziness, and tinnitus, particularly in the elderly (1). Generally, GBE has been recognized to exert beneficial pharmacological effects with few adverse reactions (2). Because elderly people frequently take various kinds of medicines, GBE–drug interactions are a major concern for the safe use of GBE. In previous studies, we found that feeding GBE to rats markedly increased the concentration of hepatic cytochrome P450 (CYP), the expression of various CYP mRNAs, and the activity of some enzymes (3, 4). Moreover, we reported that pretreatment of rats with GBE attenuated the efficacy of co-administered drugs such as tolbutamide (5). Reports of GBE–drug interactions in clinical studies are inconsistent; some showed interactions (6, 7), but others did not (8–10). It seems likely that the dose and intake period of GBE and the amount of the active substance that induces CYP are responsible for the inconsistent reports. GBE is a natural plant product, and the components in GBE might vary due to the time and place of harvest and the extraction methods used.

In our previous study using fractionated GBE samples, induction of the CYPs enzyme was observed in mice administered the terpenoid-rich fraction that is mainly bilobalide (11). To elucidate the GBE–drug interactions more clearly, further characterization of the active substance that induces hepatic CYPs is needed. In this study, we evaluated the time course of induction of hepatic CYPs by bilobalide in rats in order to ascertain how long is required for maximal induction and to determine if induction is maintained.

Powdered GBE was supplied by Tama Seikagaku-Kogyo Co. (Tokyo). The GBE contained 24.9% flavonoids and 10.6% total terpene, which consisted of 2.9% ginkgolide A, 1.4% ginkgolide B, 2.1% ginkgolide C, and 4.2% bilobalide. Bilobalide, resorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, testosterone, 6β-hydroxytestosterone, corticosterone, p-nitrophenol, 4-nitrocatechol, and 7-ethoxycoumarin were purchased from Sigma-Aldrich (St. Louis, MO, USA), (S)-Warfarin and 7-hydroxywarfarin were from Ultrafine (Manchester, England); NADPH, from Oriental Yeast (Tokyo); antibodies for mouse anti-rat CYP2B1/2B2 and horseradish peroxidase–labeled anti-mouse IgG, from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka).

Male Wistar rats (5-week-old; Japan SLC, Inc.,
Shizuoka) were kept in stainless steel wire-bottomed cages (3–4 rats in each group). They had free access to laboratory feed (CE-2; CLEA Japan, Inc., Tokyo) and tap water, and they were kept at a constant temperature (23 ± 1°C) with a 12-h light-dark cycle. Rats were given a single dose of GBE, bilobalide, or vehicle. At predetermined time points, the rats were anesthetized with pentobarbital, blood samples taken from the descending aorta using a heparinized syringe, and then the samples centrifuged to obtain the plasma. Subsequently, livers were quickly removed, weighed, and snap frozen with dry ice. A portion of the liver was stored in RNAlater (Applied Biosystems, Inc., Foster City, CA, USA) for the mRNA measurement. Plasma and liver samples were stored at −80°C until analysis.

All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the Care and Use of Laboratory Animals and were approved by an ethical committee.

Drug metabolizing enzyme activities and Western blot analysis of CYP2B enzyme in liver were performed by the previously described methods (4). Total RNA was extracted using a QuickGene RNA tissue kit SII (Fuji Photo Film Co., Ltd., Tokyo). Total RNA was subjected to the real time RT-PCR reaction using the One-Step SYBR RT-PCR kit (Perfect Real Time; Takara Bio Inc., Shiga) according to the manufacturer’s protocol and Mx3000P® (STRATAGENE Co., La Jolla, CA, USA). Cyclophilin A (Cyclo) mRNA in each sample was amplified simultaneously for an internal control. The primers were synthesized via the Perfect Real-Time Primer support system of Takara Bio.

Bilobalide was analyzed by LC-MS. Briefly, plasma or liver homogenate (200 μL) were mixed with 50 μL of 40% trichloroacetic acid and centrifuged for 20 min at 7,500 × g to precipitate the protein. The resulting supernatant (20 μL) was subjected to LC-MS analyses using an LCMS-2010EV instrument (Shimadzu Co., Kyoto). The LC conditions were as follows: column: Cadenza CD-C18 column (4.6 × 250 mm; Imtact Co., CA, USA), mobile phase: methanol-water (33:67, v/v), flow rate: 0.6 mL/min, and column temperature: 35°C. The MS conditions were as follows: probe temperature: 300°C, gas pressure: 0.02 MPa, capillary voltage: −4.0 kV, nebulizer gas flow: 2.5 L/min. Bilobalide was selectively detected by selected ion monitoring of the respective [M-H]- ions (m/z 325.1) with the scan rate at 0.1 s. Total recovery of bilobalide in plasma and liver

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**Fig. 1.** Time-dependent changes in hepatic CYP activity in rats given a single dose of bilobalide. Rats were intragastrically given a single dose of bilobalide (30 mg/kg) and sacrificed at the indicated times (2, 6, and 24 h). Each value is expressed as the mean ± S.E.M. for 3–4 rats. *P<0.05, **P<0.001: significant vs. control.
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The homogenate was about 90%, and the detection limit was 1.0 ng/mL.

The data are presented as the mean ± S.E.M. for each individual group. Statistical analyses were carried out by one-way ANOVA with Dunnett’s multiple comparison post hoc test. Differences with $P < 0.05$ were considered to be significant. These statistical tests were performed with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

The plasma bilobalide concentration in rats given an equivalent dose of approximately 40 mg/kg of bilobalide by intragastric gavage of GBE showed an almost 500-fold reduction between 2 and 24 h (mean ± S.E.M.: 2745.6 ± 248.6 and 5.661 ± 0.242 ng/mL, respectively).

At 48 h, plasma bilobalide was undetectable. A similar trend was observed in liver bilobalide concentration (mean ± S.E.M.: 6604 ± 325 ng/g at 2 h, 25 ± 8 ng/g at 24 h, and undetectable at 48 h).

Drug metabolizing enzyme was evaluated at 2, 6, and 24 h after a single dose of 30 mg/kg of bilobalide. The enzyme activity of various subtypes of CYP increased up to 24 h (Fig. 1). Amongst these, the change in pentoxyresorufin O-dealkylase for CYP2B was most pronounced. The CYP2B protein expression induced by the treatment with bilobalide was confirmed by Western blot analysis; the time course of the expression was similar to that for the enzyme activity (data not shown).

RT-PCR analysis revealed a time-dependant change in...
the mRNA expression of CYPs (Fig. 2). Interestingly, the peak induction in mRNA expression of CYP2B1, CYP2B2, and CYP3A1 occurred at 6 h and then declined at 24 h.

GBE is regarded as generally safe and well tolerated (12), and the only major safety concern is its interactions with co-administered drugs. In our previous report (11), bilobalide in GBE was shown to be a major substance responsible for the induction of CYP. In this study, the detailed characteristics of CYP enzyme induction by bilobalide were examined in rats.

The half life (t₁/₂) of bilobalide in blood is reported to be about 2 h (2). In the present study, bilobalide, especially in the liver, was clearly detected at 2 h after a single dose of GBE equivalent to approximately 40 mg/kg of bilobalide, but was considerably decreased at 24 h and undetectable at 48 h. These findings indicate that bilobalide is excreted rapidly after the administration and is not accumulated in the liver. The increase in both CYP enzyme activity and protein expression of CYP2B, which was the CYP isoform most inducible by bilobalide, was higher at 24 h than at 6 h after oral administration of bilobalide (30 mg/kg), but the CYP2B mRNA expression was maximal at 6 h and then declined. The changes in CYP2B mRNA may reflect the rapid elimination of bilobalide in plasma and liver. These findings suggest that bilobalide markedly induced CYPs, especially the 2B type, but the induction was turned off quickly due to bilobalide’s rapid elimination. We have shown previously that the continuous and excess feeding of GBE (approximately dose: 500 mg GBE/kg and 21 mg bilobalide/kg) for 1 week to rats markedly induced hepatic CYPs, but discontinuation of the treatment led to the recovery of the normal level of CYPs within 1 week (13).

GBE is a natural plant product that contains many different chemicals and is used in a variety of products with different compositions. Most of the commercially available GBE products are standardized according to the content of ginkgo flavonol glycosides (glycosidic derivatives of quercetin, kaempferol, and isorhamnetin) and terpenoids (ginkgolides A, B, C, and bilobalide), which comprise 22%–27% and approximately 5%–7% of GBE, respectively (1, 2). In a separate study, the induction of hepatic drug metabolizing enzymes was compared among four terpenoids, namely ginkgolide A, B, C, and bilobalide in mice who were orally given one of the four terpenoids for 5 days. Similar to the present rat study, bilobalide caused a marked induction of CYP enzyme, but such induction was not observed by the use of the other three terpenoids (unpublished results). A similar finding (14) reported that the inductions of CYPs in rats by ginkgolide A, B quercetin, and kaempferol were less than that caused by bilobalide. Further studies will be needed to clarify the molecular mechanism of CYPs induction by bilobalide. Overall, our novel results indicate preferential time-dependent induction of CYP2B by bilobalide.

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References

10. Wolf HR. Does Ginkgo biloba special extract EGB 761 provide additional effects on coagulation and bleeding when added to acetylsalicylic acid 500 mg daily? Drugs R D. 2006;7:163–172.