

THE LICORICE ROOT DERIVED ISOFLAVAN GLABRIDIN INHIBITS THE ACTIVITIES OF HUMAN CYTOCHROME P450S 3A4, 2B6, AND 2C9

UTE M. KENT, MICHAEL AVIRAM, MIRA ROSENBLAT, AND PAUL F. HOLLENBERG

Department of Pharmacology, the University of Michigan (U.M.K., P.F.H.) and Lipid Research Laboratory, Rambam Medical Center, Haifa, Israel (M.A., M.R.)

(Received January 14, 2002; accepted March 7, 2002)

This article is available online at <http://dmd.aspetjournals.org>

ABSTRACT:

The potent antioxidants licorice root extract and glabridin, an isoflavan purified from licorice root extract, were tested for their ability to modulate the activities of several cytochrome P450 (P450) enzymes. P450 3A4, the major human drug metabolizing P450 enzyme, was inactivated by licorice root extract and by glabridin in a time- and concentration-dependent manner. The inactivation was NADPH-dependent and was not reversible by extensive dialysis. Further analysis showed that the loss in enzymatic activity correlated with a loss in the P450-reduced CO spectrum and with a loss of the intact heme moiety. In contrast, incubations of P450 3A4 with similar concentrations of 2,4-dimethylglabridin and NADPH did not lead to inactivation of P450 3A4. P450 2B6 was also inactivated by glabridin in a time- and concentration-dependent man-

ner. The majority of the glabridin-inactivated P450 2B6 was able to form a reduced CO spectrum suggesting that the heme was not modified with this isoform. High-performance liquid chromatography analysis of the P450 heme confirmed that incubations with glabridin and NADPH did not result in the destruction of the heme moiety. The activity of P450 2C9 was competitively inhibited by glabridin, whereas P450 2D6 and P450 2E1 were virtually unaffected. The data show that glabridin can serve as a substrate for at least three human P450 enzymes and that depending on the isoform, metabolism of glabridin can lead to mechanism-based inactivation or inhibition of the P450. Heme and reduced CO spectral analysis also indicated that glabridin inactivated P450s 2B6 and 3A4 by different mechanisms.

Liver microsomal P450¹ enzymes are involved in the metabolism of endogenous substrates such as fatty acids, cholesterol, and steroids. These enzymes also carry out an important function in the catalysis and ultimate clearance of many structurally distinct xenobiotics such as drugs, pesticides, carcinogens, and environmental pollutants (Porter and Coon, 1991; Rendic and DiCarlo, 1997).

P450 3A4, the major hepatic and intestinal P450 enzyme in humans, metabolizes more than 50% of clinically used drugs such as cyclosporine A, dihydropyridines, ethynylestradiol, midazolam, terfenadine, and triazolam. On average, P450 2B6 comprises approximately 0.2% of human liver P450 and is responsible for the metabolism of roughly 3% of all drugs such as ketamine (Yanagihara et al., 2001), orphenadrine, secobarbital, phenobarbital, dexamethasone, and rifampin (Chang et al., 1997). The P450 2C family comprises approximately 18% of the P450 enzymes found in human liver and are responsible for the metabolism of at least 25% of all drugs including tolbutamide, diclofenac, (*S*)-warfarin, phenytoin, and hexobarbital. The expression of 2C enzymes is variable. Enzyme levels depend in

part on the inducibility or inactivation of this family by drugs such as rifampicin or tienilic acid. In addition, genetic polymorphisms have been observed (Rendic and Di Carlo, 1997).

Compounds such as drugs or nutrients that either compete with each other for metabolism by P450s or that inactivate P450 enzymes may thereby affect the bioavailability of certain drugs, potentially leading to severe clinical manifestations. Herbal supplements are largely unregulated, and many patients do not inform their physician of the over-the-counter supplements they consume. Therefore, drug-nutrient interactions with components in herbal supplements and clinically prescribed drugs present an increasing concern. A growing number of naturally occurring phytochemicals in foods and herbal supplements have been shown to be substrates or inactivators of P450 enzymes. Some examples include bergamottin, a component of grapefruit juice (He et al., 1998); capsaicin from chili peppers (Surh and Lee, 1995); diallylsulfide found in garlic oil (Brady et al., 1991); numerous isothiocyanates found in all cruciferous vegetables (Conaway et al., 1996; Goosen et al., 2000); I3,II8-bia-piogenin, hypericin, hyperforin, and other components of St. John's Wort (Orbach, 2000); roquefortine, a secondary cyclopeptide metabolite generated by *Penicillium roquefortine* and other *Penicillium* species (Aniant et al., 2001); resveratrol, a component of red wine (Chan and Delucchi, 2000); and thujones found in the liqueur absinthe (Höld et al., 2001).

Some dietary nutrients such as red wine flavonoids, pomegranate tannins, and licorice isoflavanes are potent antioxidants against LDL oxidation (Vaya et al., 1996; Aviram et al., 2000). LDL oxidation is considered a major risk factor for atherosclerosis (Aviram, 2000), and possible cellular sources of LDL oxidants include the NADPH oxi-

This work was supported in part by National Institutes of Health Grant CA-16954 (P.F.H.) and Rappaport Institute Research Fund (M.A.).

¹ Abbreviations used are: P450, cytochrome P450; LDL, low density lipoprotein; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; 7EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; 7BFC, 7-benzoyloxy-4-(trifluoromethyl)coumarin; reductase, NADPH-cytochrome P450 reductase.

Address correspondence to: Paul F. Hollenberg, Department of Pharmacology, University of Michigan, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0632. E-mail: phollen@umich.edu.

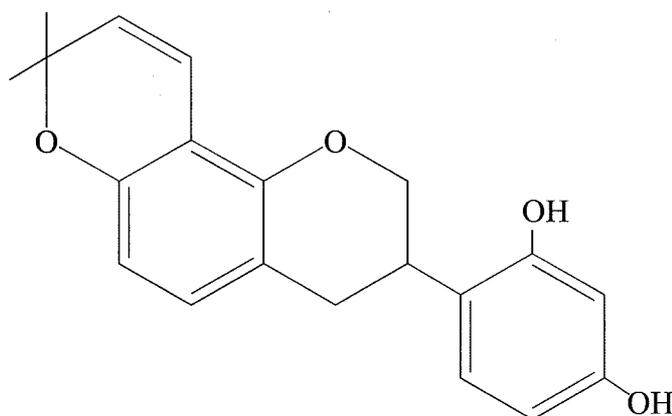


FIG. 1. Structure of glabridin.

The two hydroxyl groups on the 2 and 4 position of the B ring of glabridin are methylated in 2,4-dimethylglabridin.

dase (Aviram et al., 1996), lipoxygenase (Parthasarathy et al., 1989), myeloperoxidase (Podrez et al., 2000), paraoxanase (Aviram et al., 1999b), and the P450 enzymes (Aviram et al., 1999a). The herbal supplement licorice is reported to have "liver protective" functions (White and Foster, 2000). The dried roots of the licorice plant *Glycyrrhiza glabra* have been consumed for the past 6000 years and are used as flavoring and sweating agents, as demulcents and expectorants in the Western world and as anti-allergic and anti-inflammatory agents in Japan and China (Chandler, 1985; Mitschner et al., 1986). Flavanoid components of licorice root extract (glabridin, glabrene) were shown to have antitumorigenic, antimicrobial, antiviral, anti-inflammatory, and antioxidative activity. Licorice root extract, as well as its major flavanoid, the isoflavan glabridin, are potent antioxidants against LDL oxidation in mice and humans (Rosenblat et al., 1999). In a recent report, glabridin was also found to inhibit the activity of macrophage NADPH-oxidase, presumably by inhibiting protein kinase C (Rosenblat et al., 1999).

In the present study, the effect of glabridin (Fig. 1), obtained from an alcoholic licorice root extract, on the activities of several human P450 enzymes that play a key role in the metabolism of clinically used drugs was investigated. Glabridin was found to inactivate the enzymatic activities of P450s 3A4 and 2B6 in a mechanism-based manner requiring the presence of NADPH. In contrast, P450 2C9 was competitively inhibited but not inactivated by glabridin. These results suggest the possibility of potential drug-flavanoid interactions in individuals that consume licorice supplement in conjunction with drugs that are metabolized by P450 enzymes.

Experimental Procedures

Materials. L- α -Dilaurylphosphatidylcholine, L- α -dioleoyl-sn-glycero-3-phosphocholine, phosphatidylserine, catalase, glutathione, δ -aminolevulinic acid hydrochloride, NADPH, testosterone, and BSA were purchased from Sigma-Aldrich (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7EFC) was obtained from Molecular Probes Inc. (Eugene, OR). 7-Benzyloxy-trifluoromethylcoumarin (7BFC) was from Gentest Corp. (Woburn, MA). Licorice root extract, glabridin and 2,4-dimethylglabridin were isolated as described previously (Belinky et al., 1998).

Enzymes. Cytochromes P450 3A4, 2B6, 2E1, 2C9, and 2D6 were purified after expression in *Escherichia coli* as described previously (Gillam et al., 1993; Hanna et al., 2000). NADPH-P450 reductase was expressed in *E. coli* and purified as described by Hanna et al. (1998). Cytochrome b_5 was isolated from rat liver as described by Waxman and Walsh (1982).

Enzyme Activity Assays. The reconstitution and activity assays for P450s 3A4, 2B6, 2D6, 2C9, and 2E1 were performed essentially as previously

described (Yanev et al., 1999). P450 3A4 was reconstituted with reductase and a lipid mixture (20 μ g of a 1:1:1 mixture of L- α -dilauryl-, L- α -dioleoyl-sn-glycero-3-phosphocholine, and phosphatidylserine) for 15 min at room temperature. The reconstitution mixture contained 0.5 nmol of P450 3A4, 1 nmol of reductase, 20 μ g of lipid mixture, and 200 μ g of cholate. Following the reconstitution, the samples were brought to a total volume of 800 μ l with 50 mM Hepes buffer (pH 7.5) containing 20% glycerol, 500 U of catalase, 2 mM glutathione, 30 mM MgCl₂, and 0.5 mM EDTA. For the time- and concentration-dependent studies, aliquots of the above reconstitution mixture received licorice extract (0.0125–6.25 μ g/ml) or glabridin (0.625–40 μ M) in 1 μ l of ethanol per 85 μ l of reaction mixture. Ethanol (1 μ l) was added to 85 μ l of the control incubation. In some experiments, 40 μ M 2,4-dimethylglabridin was added. The samples were incubated at 37°C for 10 min prior to initiating the reactions with NADPH at a final concentration of 1.2 mM. Aliquots (10 pmol of P450 3A4) were removed at 0, 2, 4, 8, and 16 min, and the residual P450 3A4 activity was measured using BFC as the substrate in 0.6 ml of a secondary reaction mixture. The secondary reaction mixture contained 50 μ M BFC, 3.3 mM MgCl₂, 40 μ g of BSA/ml, and 1 mM NADPH in 200 mM potassium phosphate (pH 7.4) and was incubated for 15 min at 37°C. The reaction mixtures were quenched with 225 μ l of a mixture containing 80% CH₃CN and 20% 0.5 M Tris base in water. The hydroxylated product of 7BFC was measured spectrofluorometrically with excitation at 409 nm and emission at 530 nm. The 7BFC *O*-debenzylation activity of the control samples at 0 min in the absence of glabridin ranged from 1 to 1.9 pmol/pmol P450 3A4/min. For all assays, the activity of the control sample at 0 min in the absence of licorice or glabridin was assigned a value of 100%. The percentage of activity remaining for all other samples was calculated as a percentage of the control at 0 min.

The activity of some samples was measured using testosterone as the substrate to test if glabridin also affected the ability of P450 3A4 to metabolize testosterone (Sonderfan et al., 1987). An aliquot of the reaction mixture (50 μ l) was transferred to 950 μ l of a secondary reaction mixture containing 200 mM testosterone, 500 U of catalase, 2 mM glutathione, 30 mM MgCl₂, and 0.5 mM EDTA in 50 mM Hepes buffer (pH 7.5) and 20% glycerol. 6 β -Hydroxytestosterone was measured by reverse-phase HPLC on a C₁₈ column (4.6 \times 15 cm, 5 mm, Microsorb-MV; Rainin Instruments, Woburn, MA). Testosterone metabolites were eluted isocratically with 65% methanol at a flow rate of 1 ml/min. The eluate was monitored at 254 nm.

P450 2B6 (0.5 nmol) was reconstituted with reductase (1 nmol) and 200 μ g of L- α -dilaurylphosphatidylcholine/ml for 45 min at 4°C. The reconstitution mixture was diluted to 0.5 ml with 50 mM potassium phosphate buffer (pH 7.4) containing 110 U of catalase/ml. The samples received 0 to 100 μ M glabridin (1 μ l in ethanol/100 μ l of reaction mixture) or 1 μ l of ethanol for the control samples. The reaction mixtures were incubated for 10 min at 37°C prior to initiating the reactions with 1.2 mM NADPH. At the indicated times, aliquots (10 μ l, 10 pmol of P450 2B6) were transferred to 990 μ l of a secondary reaction mixture containing 100 μ M 7EFC, 1 mM NADPH, and 40 μ g of BSA/ml in 50 mM potassium phosphate buffer (pH 7.4) (Buters et al., 1993). The secondary reaction mixtures were incubated for 10 min at 37°C and then quenched with 334 μ l of ice-cold acetonitrile. The 7-hydroxy-4-(trifluoromethyl)coumarin product was measured spectrofluorometrically on a SLM-Aminco model SPF-500C spectrofluorometer (SLM-Aminco, Urbana, IL) with excitation at 410 nm and emission at 510 nm. The 7EFC *O*-deethylation activity of the control samples at 0 min in the absence of glabridin ranged from 0.6 to 1.3 pmol/pmol P450 2B6/min. The activity of the control sample at 0 min in the absence of glabridin was assigned a value of 100%. The percentage of activity remaining for all other samples was calculated as a percentage of the control at 0 min.

P450s 2E1, 2C9, and 2D6 were reconstituted and assayed after incubating with the indicated concentrations of glabridin using the 7EFC assay essentially as described for P450 2B6 except that for 2C9 (110 pmol in 50 μ l) the secondary reaction mixture (950 μ l) also contained 50 mM MgCl₂, and the reactions were stopped with 334 μ l of cold 0.1 M Tris (pH 9) containing 30% acetonitrile (Yanev et al., 1999). The 7EFC *O*-deethylation activity of the control samples at 0 min in the absence of glabridin was 0.3 pmol/pmol P450 2C9/min.

Spectral Analysis. For spectral analysis, P450s 3A4 and 2B6 were reconstituted as described above and incubated either with ethanol or glabridin. Exposed samples received glabridin (10 μ M for 3A4 and 30 μ M for 2B6) and

TABLE 1

Effect of licorice root extract on the activity of P450 3A4

P450 3A4 was reconstituted with reductase and lipid, incubated with licorice root extract, and evaluated for 7BFC activity as described under *Experimental Procedures*. The data shown represents the averages and ranges from duplicate samples.

Licorice Root Extract $\mu\text{g/ml}$	% Activity Remaining	
	0 min	15 min
0	100	103 \pm 1
1.4	110 \pm 7	73 \pm 2
6.9	110 \pm 1	45 \pm 1
14	79 \pm 2	25 \pm 1
69	40 \pm 3	12 \pm 2

no NADPH, whereas inactivated samples were incubated with glabridin and NADPH for 20 min (for P450 3A4) or 10 min (for P450 2B6) at 37°C. At 0 and 20 or 10 min (for P450s 3A4 or 2B6, respectively) after adding NADPH, aliquots were removed to test for enzymatic activity with 7BFC (for P450 3A4) or 7EFC (for P450 2B6). At the same times, 100 pmol of each P450 were removed and transferred into 900 μl of 50 mM potassium phosphate buffer (pH 7.4) containing 0.6% Tergitol Nonidet P-40 and 40% glycerol (quench buffer). P450-reduced CO spectra were recorded as described by Omura and Sato (1964).

HPLC Analysis. For HPLC analysis, samples containing 100 pmol of P450 3A4 or P450 2B6 were injected onto a C_4 column (4.9 \times 250 mm; Vydac/The Separations Group, Hesperia, CA) equilibrated with 40% CH_3CN , 0.1% trifluoroacetic acid. The components were resolved by increasing the percentage of CH_3CN to 80% over 30 min at a flow rate of 1 ml/min. The elution of native or modified intact heme was monitored at 405 nm and also with diode array spectroscopy from 220 to 500 nm to detect possible heme fragments. The areas under the heme peaks were integrated using the Millennium software (Waters Corp., Milford, MA). The protein components in the reaction mixture were monitored at 280 nm.

Results

Effect of Licorice Root Extract on the Activity of P450 3A4 in the Reconstituted System. The data in Table 1 suggest that an alcoholic extract of licorice root (1.4 to 69 $\mu\text{g/ml}$) inhibited the 7BFC activity of P450 3A4 in a dose-dependent manner. An activity loss of more than 50% was observed when P450 3A4 in the reconstituted system was incubated with 6.9 $\mu\text{g/ml}$ of licorice extract for 15 min in the presence of NADPH whereas no inhibition was seen at 0 time. Higher concentrations of extract (14 and 69 $\mu\text{g/ml}$) resulted in inhibition of the 7BFC activity already at 0 min compared with control incubations. Presumably this inhibition was due to carryover of the licorice root extract into the secondary BFC reaction mixture. The final concentrations of licorice root extract in the secondary reaction mixtures were 0.035, 0.173, 0.35, and 1.73 $\mu\text{g/ml}$.

The effect of increasing concentrations of licorice root extract on the 7BFC activity of P450 3A4 was examined as a function of the incubation time with NADPH. The data in Fig. 2 illustrate that the activity loss was both concentration- and time-dependent. The data also show that for the concentrations tested, inactivation occurred after an initial lag period of approximately 5 to 10 min. This observation could indicate several possibilities: 1) licorice root extract or a component in the extract has to be metabolized to a product that has to accumulate to a sufficiently high concentration and then, upon further metabolism, results in the formation of an inactivating species; or 2) one or more components in licorice root extract may be metabolized by P450 3A4, and the ones that are turned over most readily need to be removed by metabolism before the inactivating compound can be enzymatically converted to a reactive intermediate.

Effect of Glabridin on the BFC Activity of P450 3A4 in the

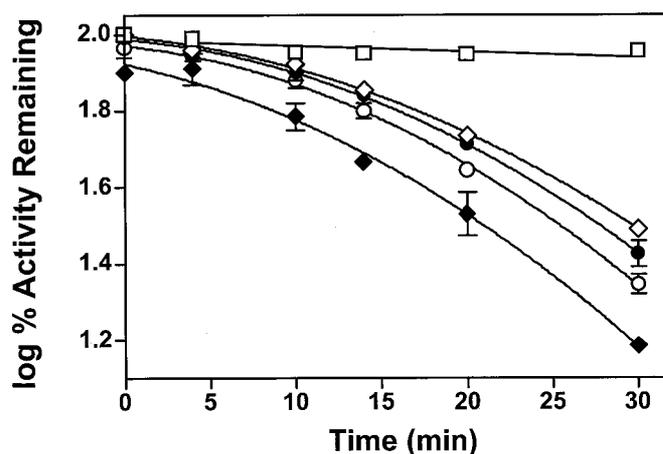


FIG. 2. Effect of licorice root extract on the 7BFC *O*-debenzylation activity of P450 3A4.

P450 3A4 was reconstituted and incubated with licorice root extract as described under *Experimental Procedures*. At the indicated time points, samples were removed and assayed for activity remaining. The amounts of the extract added were (\square) 0, (\diamond) 0.0125 $\mu\text{g/ml}$, (\bullet) 0.125 $\mu\text{g/ml}$, (\circ) 1.25 $\mu\text{g/ml}$, and (\blacklozenge) 6.25 $\mu\text{g/ml}$. Each data point represents the average and the error from two separate experiments.

Reconstituted System. Based on the results obtained with the licorice root extract, glabridin, (Fig. 1) a purified component of this extract, was examined for its ability to inactivate P450 3A4. Initial observations with 50 μM glabridin showed that both the 7BFC *O*-debenzylation activity and the testosterone hydroxylation activity of P450 3A4 were completely inactivated following 15 min of incubation in the presence of NADPH (data not shown). Since the 7BFC *O*-debenzylation activity is easier to measure and faster to perform than the testosterone assay, the 7BFC assay was used to assess activity losses in all subsequent experiments. The data in Fig. 3 show the loss in P450 3A4 activity following incubation with different concentrations of glabridin. The kinetic constants describing the inactivation were derived from the inset to Fig. 3. The rate of inactivation at 37°C was 0.14 min^{-1} . The K_I for inactivation was 7 μM , and the time required for the P450 3A4 activity to decrease by 50% was 5 min. Incubations with 5 to 40 μM of glabridin resulted in initial losses in the BFC activity at 0 min. At these concentrations, the carryover of glabridin into the secondary reaction mixture was 0.125 to 1 μM . Presumably this amount of glabridin was available for metabolism in the secondary reaction mixture and contributed to the activity loss observed at 0 min.

Reversibility of Inactivation. Table 2 shows that the inactivation of P450 3A4 by glabridin was not reversible when inactivated and control samples were dialyzed over night to remove free or bound glabridin. The minor increase in activity of the dialyzed samples compared with control samples probably reflects the removal of noncovalently bound glabridin. Addition of fresh reductase to the dialyzed samples also did not result in a substantial recovery of the enzymatic activity, suggesting that the loss in activity occurred because of inactivation of P450 3A4 and not reductase.

Effect of Glabridin on P450 3A4 Activity and Reduced CO Spectrum. The loss in P450 3A4 activity was dependent on carrying out the incubation with glabridin in the presence of NADPH (Table 3). Samples incubated with glabridin but without NADPH showed no loss in activity or in the amount of the P450-reduced CO spectrum when compared with control incubations carried out in the absence of glabridin. When samples were incubated together with 10 μM glabridin and NADPH, a 54% loss in enzymatic activity with a concurrent 42% loss in the P450-reduced CO spectrum was seen. When P450

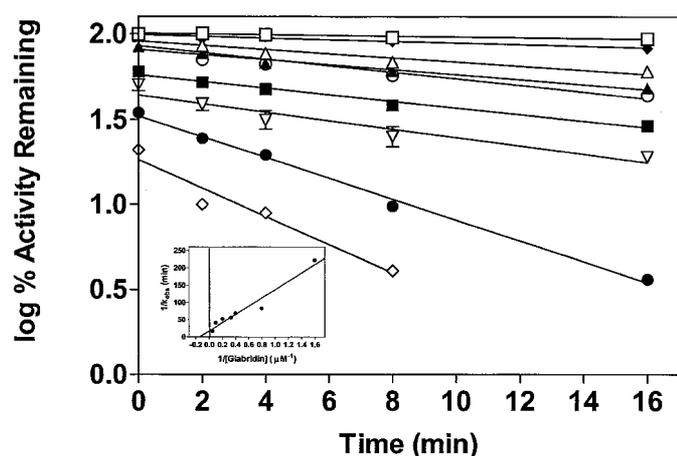


Fig. 3. Effect of glabridin on the 7BFC *O*-debenzoylation activity of P450 3A4.

P450 3A4 was reconstituted and incubated with glabridin as described under *Experimental Procedures*. At the indicated time points, samples were removed and assayed for residual activity remaining. The concentrations of glabridin were (□) 0 μM , (◇) 0.625 μM , (△) 1.25 μM , (▲) 2.5 μM , (○) 3.0 μM , (■) 5.0 μM , (▽) 10 μM , (●) 20 μM , and (◇) 40 μM . The inset shows the double-reciprocal plot of the rates of inactivation of 7BFC *O*-deethylation activity as a function of the glabridin concentration. The data represent the mean and standard deviations from four separate experiments. The standard deviations for most points were smaller than the size of the symbol.

TABLE 2

Effect of dialysis and fresh reductase on the activity of P450s 3A4 and 2B6 inactivated with glabridin

P450s 3A4 and 2B6 were reconstituted with reductase and lipid, incubated with 10 or 30 μM glabridin for 10 or 30 min, respectively, and evaluated for 7BFC or 7EFC activity followed by dialysis as described under *Experimental Procedures*. The data represent the mean and standard deviation of 2 to 4 samples from duplicate experiments.

P450 Sample	% Activity Remaining	
	Control	Inactivated
3A4 before dialysis	100	40 ± 2
3A4 after dialysis	100	57 ± 3
3A4 after dialysis + fresh reductase	100	67 ± 2
2B6 before dialysis	100	20 ± 1
2B6 after dialysis	100	34 ± 1
2B6 after dialysis + fresh reductase	100	33 ± 3

3A4 samples were incubated with higher concentrations of glabridin (30 μM), a greater loss in activity with a concurrent loss in the P450-reduced CO spectrum was seen.

HPLC and Diode Array Analysis of P450 3A4 and Glabridin-Inactivated P450 3A4 Heme. A loss in the P450 CO-reduced spectrum may have resulted as a consequence of heme alkylation or heme destruction by a glabridin reactive intermediate. However, it has been shown that a loss in the CO-reduced spectrum may also occur without destruction of the heme (Kent et al., 2001). Therefore, P450 3A4 samples that either were exposed to glabridin or inactivated with glabridin were examined using HPLC separation and diode array detection from 220 to 500 nm. The diode array spectrum of the heme moiety from glabridin-exposed control or glabridin-inactivated P450 3A4 samples was identical in both spectra showing a maximal absorbance at 405 nm for the intact heme moiety (data not shown). When the area under the heme peaks (detected at 405 nm) from control and inactivated samples were compared, a loss of heme was observed in all inactivated samples that was comparable to the loss in activity (Table 4). No additional peaks eluting at later times with an absorbance at 405 nm indicative of glabridin-modified heme products were found. This observation suggested that some destruction of the heme

had occurred resulting in a loss of spectrally detectable heme at 405 nm.

Effect of 2,4-Dimethylglabridin on the P450 3A4 Activity. Unlike glabridin, the 2,4-dimethyl derivative of glabridin is not an antioxidant. When P450 3A4 was incubated with 40 μM 2,4-dimethylglabridin no loss in activity was observed (open triangles) (Fig. 4). In contrast, incubations with 40 μM glabridin again resulted in inactivation of the enzyme.

Effect of Glabridin on the 7EFC Activity of P450 2B6. Purified P450 2B6 in the reconstituted system was also inactivated by glabridin in a time- and concentration-dependent manner (Fig. 5). The kinetic constants describing the inactivation event were derived from the inset to Fig. 5. The concentration of glabridin required to obtain half-maximal inactivation was 12 μM . The rate of inactivation at 37°C was 0.08 min^{-1} , and the time required for one-half of the P450 2B6 7EFC *O*-deethylation activity to disappear was 8.3 min. A loss in activity at 0 min was again observed when the concentrations of glabridin in the primary reaction mixture were greater than 50 μM . Presumably this initial activity loss was due to the carryover of glabridin into the secondary 7EFC reaction mixture allowing for further metabolism of glabridin and additional inactivation of some of the P450 2B6 molecules. The concentration of glabridin in the secondary reaction mixture was between 0.5 and 1 μM .

Effect of Glabridin on P450 2B6 Activity, Reduced-CO Spectrum, and Heme Retention. The inactivation of P450 2B6 by glabridin required a catalytic step since the loss in activity was dependent on carrying out the incubations in the presence of NADPH (Table 3). Samples incubated with glabridin but without NADPH showed no time-dependent loss in activity or significant loss in the P450-reduced CO spectrum when compared with control incubations carried out in the absence of glabridin. When P450 2B6 samples were incubated together with 30 μM glabridin and NADPH, a 63% loss in enzymatic activity was seen. However, unlike the results obtained with P450 3A4, most of the P450 2B6 was able to form a reduced CO complex. Similarly, HPLC separation followed by diode array detection of the P450 2B6 heme showed that glabridin-inactivated samples, which had lost 80% in activity, lost only 18% of the detectable heme at 405 nm (Table 4).

Reversibility of Inactivation. Table 2 shows that the inactivation of P450 2B6 by glabridin was not reversible by overnight dialysis to remove free or noncovalently-bound glabridin. As with inactivated P450 3A4, the addition of fresh reductase to the dialyzed samples did not lead to a significant recovery of the enzymatic activity, again suggesting that the inactivation event occurred because of a modification on the P450 and not the reductase.

Effect of Glabridin on the Activities of P450s 2C9, 2D6, and 2E1. With P450 2C9, no significant time-dependent loss indicative of mechanism-based inactivation was observed. However, the activity of purified P450 2C9 in the reconstituted system was inhibited by 1, 10, and 100 μM glabridin in a concentration-dependent manner with 50% inhibition occurring at approximately 100 μM glabridin (Fig. 6). The activity of P450 2D6 was unaffected at concentrations of 1 or 10 μM glabridin and slightly inhibited (approximately 15%) when the concentration of glabridin was increased to 100 μM (data not shown). P450 2E1 was not affected when incubated with glabridin and NADPH at the same concentrations used for P450s 2C9 and 2D6 (data not shown).

Discussion

The use of herbal supplements has been widespread in Europe and Asia for many centuries, and there is also a more recent trend toward using natural remedies in the United States. Since these supplements

TABLE 3

Effect of glabridin on the catalytic activity and P450 reduced CO spectrum of P450s 3A4 and 2B6

P450s 3A4 and 2B6 were reconstituted with reductase and lipid, incubated with 10 or 30 μM glabridin, respectively, and evaluated for 7BFC activity and P450-reduced CO spectra as described under *Experimental Procedures*. The data shown represents the mean and standard deviation from three experiments.

P450 Incubation Conditions	% Activity Remaining			% CO Spectrum Remaining		
	0 min	15 min	30 min	0 min	15 min	30 min
3A4 - Glabridin + NADPH	100	97 \pm 2	98 \pm 4	100	86 \pm 2	95 \pm 7
3A4 + Glabridin - NADPH	94 \pm 3	95 \pm 3	79 \pm 13	99 \pm 2	93 \pm 3	87 \pm 6
3A4 + Glabridin + NADPH	94 \pm 3	36 \pm 1	37 \pm 15	99 \pm 2	58 \pm 1	85 \pm 6
2B6 - Glabridin + NADPH	100		100			
2B6 + Glabridin - NADPH	83 \pm 17		102 \pm 10			
2B6 + Glabridin + NADPH	83 \pm 17		102 \pm 10			

TABLE 4

Effect of glabridin on the activity and P450 heme of P450s 3A4 and 2B6

P450s 3A4 and 2B6 were reconstituted with reductase and lipid and incubated with 10 or 30 μM glabridin, respectively, as described under *Experimental Procedures*. Samples were removed and assayed for activity and for heme by reverse-phase HPLC with diode array detection at 405 nm. The data shown represent the mean and standard deviation from three to four experiments.

P450 Incubation Conditions	% Activity Remaining			% Heme Remaining	
	0 min	15 min	30 min	15 min	30 min
3A4 - Glabridin + NADPH	100	98 \pm 1	105 \pm 3	100	100
3A4 + Glabridin + NADPH ^a	84 \pm 2	47 \pm 10	20 \pm 1	60 \pm 2	87 \pm 6
3A4 + Glabridin + NADPH ^a	84	22		30	
2B6 - Glabridin + NADPH	100				
2B6 + Glabridin + NADPH	59 \pm 2				

^a The data shown is from separate experiments using different concentrations of glabridin. Samples were incubated either with 10 μM glabridin (first data set) or 30 μM glabridin (second data set from one experiment).

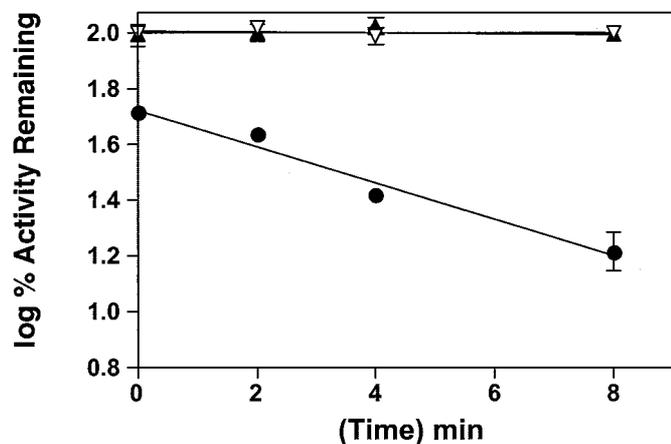


FIG. 4. Effect of glabridin and 2,4-dimethylglabridin on the 7BFC activity of P450 3A4.

P450 3A4 was reconstituted and incubated with glabridin as described under *Experimental Procedures*. At the indicated time points, samples were removed and assayed for residual activity remaining. The samples were incubated with (∇) ethanol, (\bullet) 40 μM glabridin, or (\blacktriangle) 40 μM 2,4-dimethylglabridin. The data shown represent the mean and standard deviation from three separate experiments.

are largely unregulated and have not been systematically tested, there is some concern as to the effect of simultaneous consumption of herbal and clinically used drugs (Chang, 2000). With the exceptions of grapefruit juice and St. John's wort extract, relatively little is known about potential drug-nutrient interactions of herbal or food supplements (Schmiedlin-Ren et al., 1997, Orbach, 2000). To address this issue, the effects of glabridin on the activities of human P450s in the reconstituted system were examined. Glabridin is the major ethanol extractable component and the most potent antioxidant against LDL oxidation found in licorice root. Licorice root extract and glabridin were found to inactivate P450 3A4 in a time-, concentration-,

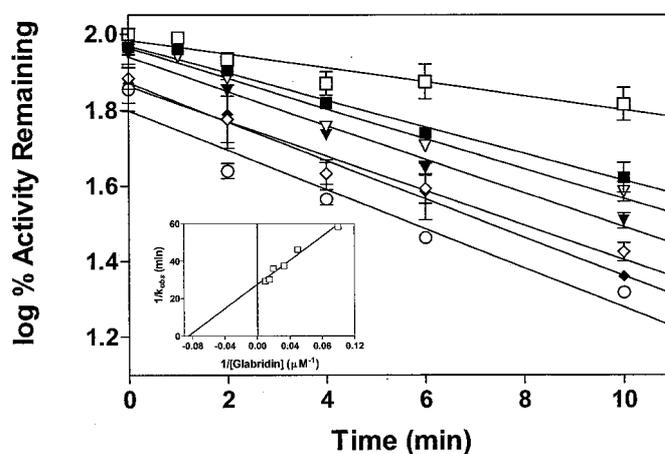


FIG. 5. Effect of glabridin on the 7EFC O-deethylation activity of P450 2B6.

P450 2B6 was reconstituted and incubated with glabridin as described under *Experimental Procedures*. At the indicated time points, samples were removed and assayed for residual activity remaining. The concentrations of glabridin were (\square) 0 μM , (\blacksquare) 10 μM , (∇) 20 μM , (\blacktriangledown) 30 μM , (\diamond) 50 μM , (\blacklozenge) 75 μM , and (\circ) 100 μM . The inset shows the double-reciprocal plot of the rates of inactivation of the 7EFC O-deethylation activity as a function of glabridin concentration. The data shown represent the mean and standard deviation from three to four separate experiments. The standard deviations for some points were smaller than the size of the symbol.

and NADPH-dependent manner, indicative of mechanism-based inactivation. Metabolism of glabridin by P450 3A4 resulted mainly in the destruction of the heme moiety since the loss in activity with different concentrations of glabridin correlated well with the loss in the P450 3A4-reduced CO spectrum and HPLC detectable heme at 405 nm. No other peaks indicative of glabridin-alkylated heme eluting after the native heme were detected at 405 nm. Presumably glabridin metabolism generated reactive intermediates that resulted in heme fragmentation. Incubations with 2,4-dimethylglabridin did not lead to

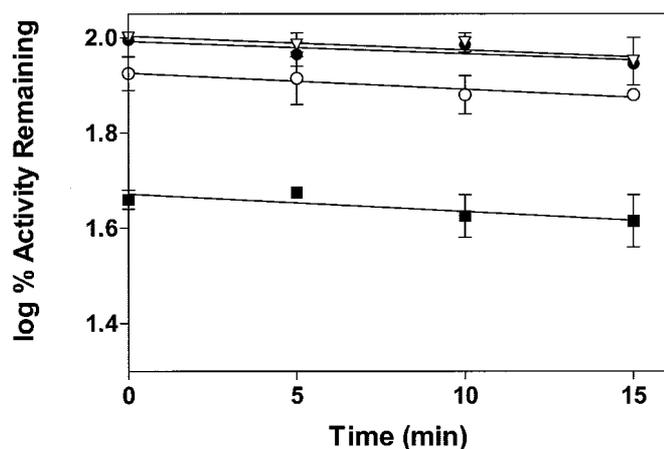


FIG. 6. Effect of glabridin on the activity of P450 2C9.

P450 2C9 was reconstituted and incubated with glabridin as described under *Experimental Procedures*. At the indicated time points, samples were removed and assayed for activity remaining. The concentrations of glabridin were (▽) 0 μ M, (●) 1 μ M, (○) 10 μ M, and (■) 100 μ M. The data shown represent the average and error from two separate experiments.

a loss in the enzymatic activity of P450 3A4. This observation could be the result of an inability of the methylated compound to bind or be metabolized. Alternatively, 2,4-dimethylglabridin could be metabolized to products that were not capable of inactivating P450 3A4. Neither glabridin nor the 2,4-dimethylglabridin was able to elicit a binding spectrum with P450 3A4 making spectral analysis of binding not feasible. Incubation of glabridin or 2,4-dimethylglabridin with P450 3A4 and NADPH in the reconstituted system for 30 min followed by extraction, HPLC separation, and diode array detection indicated that there was a decrease in the glabridin parent compound and an appearance of glabridin-related metabolites when compared with control samples. In contrast, no significant decrease in the 2,4-dimethylglabridin or the appearance of 2,4-dimethylglabridin metabolites was observed under similar conditions (data not shown). This suggested that the two hydroxyl groups on the 2' and 4' position of the flavenoid B ring of glabridin, which are believed to be essential for its antioxidative activity (Belinky et al., 1998), are also required for P450 3A4 inactivation.

The P450 2B6 7EFC activity also was inactivated by glabridin with characteristics indicative of mechanism-based inactivation. The inactivation was time- and concentration-dependent and required the presence of both NADPH and glabridin. However, little loss in the heme of P450 2B6 was observed when the reduced CO binding spectrum or the heme of the inactivated sample was measured and compared with the noninactivated controls. As was observed with glabridin-inactivated P450 3A4 samples, the inactivation of P450 2B6 was not reversible by dialysis and was not due to modification of the reductase. Presumably metabolism of glabridin by P450 2B6 generated a reactive intermediate that bound to the apoprotein. Liquid chromatography-mass spectrometry analysis of the glabridin-modified P450 2B6 did not yield any useful information about this putative adducted apo-protein. This result was not unexpected since in many instances adducted P450 2B6 could not be analyzed because it aggregated and either failed to ionize or transfer into the vapor phase (unpublished observations).

The 7EFC activity of P450 2C9 was also affected by glabridin. With this P450 isoform, a concentration-dependent loss in activity indicative of enzyme inhibition was observed. No change in the 7EFC activities of P450s 2D6 and 2E1 was observed when these enzymes were incubated with glabridin in the presence of NADPH.

Licorice root extract and its major flavonoid antioxidant glabridin seem to have a variety of beneficial effects on cells, presumably due to their antioxidative capacity (Rosenblat et al., 1999; Aviram, 2001). The current findings now also indicate that glabridin and possibly other components of alcoholic licorice root extract inactivate or inhibit the activities of some human P450 enzymes *in vitro*. These effects on P450 enzymes may play a role in the reported antiatherosclerotic activity of glabridin. Furthermore, these effects on P450 enzymes may indicate the possibility for drug-nutrient interactions, particularly in cases in which irreversible inactivation of the enzyme occurs.

Acknowledgments. We greatly appreciate the help of Chitra Sridar with the purification of P450s 3A4, 2C9, and 2D6.

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