Short Communication

OXIDATION OF THE FLAVONOIDS GALANGIN AND KAEMPFERIDE BY HUMAN LIVER MICROSONES AND CYP1A1, CYP1A2, AND CYP2C9

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ABSTRACT:

There is very limited information on cytochrome P450 (P450)-mediated oxidative metabolism of dietary flavonoids in humans. In this study, we used human liver microsomes and recombinant P450 isoforms to examine the metabolism of two flavonols, galangin and kaempferide, and one flavone, chrysin. Both galangin and kaempferide, but not chrysin, were oxidized by human liver microsomes to kaempferol, with $K_m$ values of 9.5 and 17.8 $\mu$M, respectively. These oxidations were catalyzed mainly by CYP1A2 but also by CYP2C9. Consistent with these observations, the human liver microsomal metabolism of galangin and kaempferide were inhibited by the P450 inhibitors furafylline and sulfaphenazole. In addition, CYP1A1, although less efficient, was also able to oxidize the two flavonoids. Thus, dietary flavonoids are likely to undergo oxidative metabolism mainly in the liver but also extrahepatically.

Flavonoids are polyphenolic compounds present ubiquitously in fruits, vegetables, and beverages such as tea and red wine (Hertog et al., 1992, 1993b). Flavonoids have been implicated to be protective against coronary heart disease (Hertog et al., 1993a; Knekt et al., 1997; Le Marchand et al., 2000). The bioavailability of flavonoids in human blood is mediated by extensive metabolism (Walle et al., 1997; Walle and Knekt, 1997; Le Marchand et al., 2000). The bioavailability of the dietary flavonoids, however, seems to be low, limited both by poor transport (Walgren et al., 1998) and by extensive metabolism (Walle et al., 1999). The metabolism seems to be mediated by conjugative and oxidative enzymes.

Many studies have shown potent inhibition of cytochrome P450 (P450)1 oxidation by flavonoids, in particular the CYP1A1/1A2 isoforms (Tsyrlov et al., 1994; Lee et al., 1998; Zhai et al., 1998; Moon et al., 2000). Flavonoids have also demonstrated potent interactions with the aryl hydrocarbon receptor (Cioliino and Yeh, 1999; Cioliino et al., 1999; Ashida et al., 2000). Much less is known about the oxidative metabolism of the flavonoids, in particular in humans. Studies with liver microsomes from Aroclor 1254-induced rats demonstrate extensive oxidative metabolism of several flavonoids (Duarte Silva et al., 1997a; Nielsen et al., 1998). This oxidation may be mediated by CYP1A1, as supported by subsequent studies (Duarte Silva et al., 1997b; Doostdar et al., 2000). In a recent study of chrysin (5,7-dihydroxyflavone; Fig. 1), we found no evidence of oxidation of this compound either by uninduced rat hepatocytes or in human intestinal and hepatic cell lines with induced CYP1A1 (Galijatovic et al., 1999). Surprisingly, there have been no studies of human liver microsomal metabolism of flavonoids.

To gain a better understanding of the role of human P450-mediated metabolism of the flavonoids, we investigated the metabolism of two flavonols (3-hydroxylated flavones) [i.e., galangin and kaempferide (Fig. 1)] and chrysin by human liver microsomes and recombinant P450 isoforms. In contrast to chrysin, both galangin and kaempferide underwent extensive oxidative metabolism to kaempferol (Fig. 1). This oxidation was catalyzed not only by both CYP1A1 and CYP1A2 but also by CYP2C9.

Experimental Procedures

Materials. Chrysin, furafylline, $\alpha$-glucose 6-phosphate (G6P), G6P dehydrogenase, kaempferol, and NADP were purchased from Sigma Chemical Co. (St. Louis, MO). Galangin and trifluoroacetic acid (spectrophotometric grade) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Kaempferide was purchased from Indofine Chemical Company, Inc. (Belle Mead, NJ), and sulfaphenazole was obtained from Sigma/RBI (Natick, MA). Pooled human liver microsomes from 11 donors and microsomes containing lymphoblastoid expressed human CYP1A1, 1A2, 2C9 (Arg 144), and 3A4 and baculovirus expressed human CYP3A4 (Supersomes) were obtained from GENTEST (Woburn, MA).

Flavonoid Incubations with Human Liver Microsomes and Recombinant Enzymes. Galangin, kaempferide, and chrysin (1–100 $\mu$M) were each incubated with pooled human liver microsomes from 11 donors (50 $\mu$g of protein) in a final volume of 100 $\mu$L of 100 mM sodium phosphate buffer, pH 7.6, containing 0.5 mM NADP, 10 mM MgCl$_2$, 10 mM G6P, and G6P dehydrogenase (1 unit) at 37°C for 30 min. Recombinant human CYP1A1, 1A2, 2C9, and 3A4 (25 $\mu$g of protein) were incubated with the flavonoids under identical conditions, using substrate concentrations of 1 to 100 $\mu$M.

Inhibition of CYP1A2 and 2C9 in the pooled human liver microsomes used identical incubation conditions with 10 $\mu$M concentrations of the flavonoid substrates. Furafylline (20 $\mu$M), a selective CYP1A2 inhibitor (Sesardic et al., 1990; Clarke et al., 1994; Clement and Demesmaeker, 1997) was preincubated for 15 min with the enzyme mixture before the addition of substrate. Sulfaphenazole (25 $\mu$M), a selective inhibitor of CYP2C9 (Eagling et al., 1998; Giancarlo et al., 2001), was added together with the substrates.

HPLC Analysis. The incubation mixtures were subjected to solid-phase extraction using Oasis HLB C$_{18}$ 1–ml extraction cartridges (Waters, Milford, MA), as previously described (Galijatovic et al., 1999). The dried methanol eluates were reconstituted in a mobile phase for HPLC analysis. A Symmetry C$_{18}$ column (3.9 $\times$ 150 mm; Waters) with a $\mu$Bondapak C$_{18}$ Guard-Pak...
precolumn insert (Waters), and a mobile phase of 60% methanol (galangin and kaempferide) or 55% methanol (chrysin) in 0.3% trifluoroacetic acid at a flow rate of 0.9 ml/min was used. Detection was by photodiode array (Waters model 996 detector, Millennium software), monitoring galangin and kaempferide and their metabolites at 365 nm and chrysin and its metabolites at 268 nm. The identity of possible metabolites was confirmed by a comparison of retention time and UV spectrum with synthetic standards.

Data Analysis.

All data are reported as means ± S.E. The apparent enzyme kinetic parameters $K_m$ and $V_{max}$ were obtained from the Henri-Michaelis-Menten equation by nonlinear regression analysis of velocity versus substrate concentration plots, using the Solver function of Microsoft Excel (Microsoft, Redmond, WA).

Results

Both galangin and kaempferide, but not chrysin, were oxidized by pooled human liver microsomes from 11 donors, as evidenced by the disappearance of substrates and appearance of metabolites. The product formed from both galangin and kaempferide was kaempferol, which is based on the comparison of the HPLC retention time and characteristic UV spectrum with the synthetic compound. Galangin was thus oxidized in the 4'-position, whereas kaempferide was O-demethylated. The velocity of these reactions is depicted in Fig. 2A, demonstrating the mean values for four determinations with the same pooled microsomes. The velocity versus substrate concentration curves were virtually identical for galangin and kaempferide. The resulting apparent enzyme kinetic parameters with $K_m$ values of 9.5 ± 0.4 and 17.8 ± 3.5 μM ($n$ = 4), respectively, and similar $V_{max}$ values are shown in Table 1. In sharp contrast, the flavone chrysin was not metabolized by the pooled human liver microsomes.

The P450 isoforms most likely to be involved in the metabolism of these flavonoids, based on previous observations, were primarily CYP1A1 and also CYP1A2. Indeed, both of these isoforms catalyzed the oxidation of galangin and kaempferide, as seen in Fig. 2B, showing the mean values of duplicate samples in two independent experiments. Surprisingly, CYP1A2 was considerably more efficient than CYP1A1, as also reflected in the resulting apparent enzyme kinetic parameters in Table 1. Although the $K_m$ value for oxidation of galangin by CYP1A1 was considerably lower than that of kaempferide, the $V_{max}$ values for the oxidation of the two flavonols by both CYP1A1 and 1A2 were almost identical. As with the human liver microsomes, chrysin was not metabolized by either CYP1A1 or 1A2.

For hepatic metabolism of the flavonoids, only CYP1A2 would be expected to be important, whereas CYP1A1 should mainly be considered for extrahepatic metabolism (Guengerich, 1995). To determine the potential contribution of other P450 isoforms to the hepatic metabolism of galangin and kaempferide, we used the CYP1A2-specific inhibitor furafylline in further experiments with the pooled human liver microsomes.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>HLM</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP1A1</th>
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<tr>
<td>$K_m$ (μM)</td>
<td></td>
<td></td>
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<tr>
<td>Gal</td>
<td>9.5 ± 0.4</td>
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<tr>
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</table>
concentration of 20 μM, produced about 40% inhibition (P < 0.05) of the oxidation of both substrates to kaempferol. Although this supported the importance of CYP1A2 in the human liver microsomal metabolism of these flavonoids, it also suggested that other hepatic P450 isoforms may be involved.

Based on the three-dimensional quantitative structure-activity relationship for ligands of CYP2C9 (Jones et al., 1996), this isoform was likely to use flavonoids as substrates. Indeed, both galangin and kaempferide were substrates for CYP2C9 (Fig. 2B). In contrast to CYP1A1- and CYP1A2-mediated oxidation, which was relatively similar for the two flavonols, the oxidation of galangin and kaempferide by CYP2C9 was quite different, as reflected by the apparent enzyme kinetic parameters in Table 1. Galangin had a much lower \( K_m \) value (0.4 μM) than kaempferide (8.1 μM), whereas the \( V_{\text{max}} \) value was almost 2-fold higher for kaempferide. Chrysin was not a substrate for CYP2C9. We also examined the effect of the CYP2C9-selective inhibitor sulfaphenazole (Eagling et al., 1998; Giancarlo et al., 2001) on the oxidation of galangin and kaempferide by human liver microsomes. With 25 μM sulfaphenazole, there was a small (35%) inhibition, which was statistically significant (P < 0.05).

The potential contribution of the major hepatic P450 isoform (i.e., CYP3A4) was also investigated. There was no evidence for oxidation of galangin, kaempferide, or chrysin by this isoform.

**Discussion**

This is the first study of human liver microsomal metabolism of flavonoids. It demonstrates efficient oxidation of the two flavonols galangin and kaempferide by ring oxidation and O-demethylation, respectively, to the common product kaempferol. It also identifies CYP1A2 as the main P450 isoform involved in these very similar oxidations. In addition, this study adds CYP2C9 as a contributor. The latter is in keeping with the known structure-activity relationships for substrates of this isoform (Jones et al., 1996). The isoform that had been thought to be the main isoform involved in the metabolism of flavonoids (i.e., CYP1A1) was considerably less efficient but emphasizes that extrahepatic metabolism can occur. Why chrysin, lacking the hydroxy group in the 3-position, is not oxidized by any of the preparations used has no immediate explanation. It is efficiently oxidized by microsomes from Aroclor 1254-induced rats but not from uninduced rats (Nielsen et al., 1998; Galijatovic et al., 1999), suggesting that Aroclor 1254 induces a P450 isoform not normally present in either human or rat liver.

Although oxidative metabolism of flavonoids may facilitate their elimination, it may be considered a bioactivation reaction as well. For example, the oxidation of galangin to kaempferol, which can proceed further to quercetin (Duarte Silva et al., 1997a,b), yields increasingly active molecules with regard to antioxidant properties (Piatta, 2000; Rice-Evans, 2001; Yang et al., 2001). Our observations suggest that such reactions can occur not only in the liver but also extrahepatically. The finding that CYP1A2 and CYP2C9 are major players in the metabolism of galangin and kaempferide also suggests potential interactions between flavonoids and drugs using these isoforms for their inactivation. The importance of the oxidative pathways in the metabolism of flavonoids, however, cannot be fully understood until we know more about metabolism in intact human hepatocytes or in vivo, where competing conjugation reactions occur. This is currently under investigation (Otake and Walle, 2001).

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**References**


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