Grapefruit Component Interacting with Rat and Human P450 CYP3A: Possible Involvement of Non-Flavonoid Components in Drug Interaction

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Active components in grapefruit juice, which modulate a cytochrome P450 (CYP3A) activity, were investigated. CYP3A-catalyzed 6β-hydroxylation of testosterone in livers of rat and human was inhibited by the addition of an ethyl acetate-extract of grapefruit juice. Several components of grapefruit juice, including naringin, naringenin, limonin and obacunone, also showed inhibitory effects in human liver microsomes. However, the amounts of these components in grapefruit juice are too low to account for the inhibition by the ethyl acetate-extracts. Analyses with HPLC indicate the existence of inhibitory components in the extract, which are distinct from these known compounds and are specific to grapefruit juice. These results suggest that hydrophobic components other than flavonoids, probably coumarin derivatives, are responsible for the inhibitory effect of grapefruit juice.

Key words grapefruit juice; cytochrome P450; flavonoid; CYP3A4; human liver; drug interaction

Diet is one of the major modulating factors in altering drug efficacy in humans. Bailey et al. 2, 3 reported that the oral, but not parenteral, pharmacological effect and bioavailability of a Ca 2+ channel blocker, felodipine, were increased in humans simultaneously given grapefruit juice. The increase was selective with grapefruit juice, and was not observed with other beverages such as orange and grape juices. 2, 4-10 Grapefruit juice is now known to enhance the bioavailability of several clinically important drugs such as nifedipine, 11, 12 cyclosporin, 13, 14 terfenadine, 15 ethinylestradiol, 16 midazolam, 17 and triazolam. 18 These chemicals differ from each other in their pharmacological properties and chemical structures, but share two common features. The absolute bioavailabilities of these drugs were estimated, in spite of their good intestinal absorption, to be 5 to 30%. Several studies using in vitro tissue preparations also showed that, among cytochrome P450 forms, CYP3A4 mainly mediated the oxidative metabolism of these drugs in humans. 10 CYP3A4 is expressed mainly in the liver, but is also detected in the small intestine. 19 In addition, a large interindividual difference, up to 30-fold in the hepatic levels, is observed, 10, 11 although the cause of the difference remains unclear. These results suggest the involvement of first pass metabolism in the liver and/or small intestine for the enhanced bioavailability of these drugs caused by the ingestion of grapefruit juice.

Grapefruit contains several different flavonoids as glycosides. Among their aglycones, naringenin, quercetin, kaempferol, hesperitin and apigenin were reported to inhibit microsomal CYP3A-mediated oxidation of drugs such as nifedipine in rat and human livers. 12-15 These flavonoids may thus be able to alter the pharmacokinetics of dihydropyridines in humans. Studies in vivo, however, showed no clear change in felodipine or nisoldipine pharmacokinetics in humans coadministered with naringin, 16, 17 which was contained as the major flavonoid component in grapefruit juice. From grapefruits, hydrophobic components have been isolated as bitter principles. Vasoactive components such as urodiolene 18 in grapefruit juice may affect the metabolic clearance of dihydropyridines through a change in liver blood flow. The results described above, however, suggest that an unknown component, probably other than flavonoids, in grapefruit juice is responsible for the drug interaction.

CYP3A P450 forms catalyze the oxidation of endogenous steroids such as testosterone, 19, 20 progesterone, 21 and estradiol. 22 The microsomal 6β-hydroxylation of testosterone is selectively mediated by this subfamily of P450. Therefore, we have used human liver microsomes to detect specific components in grapefruit juice which affect the CYP3A-mediated metabolism of testosterone in the present study. The results obtained indicate that specific components bearing coumarin structures in grapefruit juice have clear inhibitory effects on microsomal CYP3A4-mediated oxidation in human livers.

MATERIALS AND METHODS

Materials Grapefruit juices were purchased from local commercial sources (originated in U.S.A.). Naringin, naringenin and 11α-hydroxyprogesterone were purchased from Sigma Chemical Co. (St. Louis, MO). NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast, Ltd., and testosterone was from Nakalai Tesque, Inc. (Kyoto, Japan).

Grapefruit Juice Extract Grapefruit juices from three different commercial sources (products of Florida, U.S.A., 50 ml) were each extracted with ethyl acetate (150 ml). The organic residue after evaporation was dissolved in methanol (1 ml) and added to an incubation mixture.

Preparation of Liver Microsomes Microsomes were prepared from the livers of rats and humans as described previously. 11, 23 Male Sprague-Dawley rats (body weight, 250-300 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Hepatic microsomes were stored at ~80 °C until use. Protein was determined by a Bio-Rad protein assay dye reagent concentrate (Hercules, CA).

Assay of the Microsomal Testosterone Oxidation A typical reaction mixture (0.5 ml) consisted of 100 μM Tris-HCl buffer (pH 7.4), liver microsomes (0.1 mg of liver proteins/mg of microsomal protein, 0.5 μM testosterone, 1 μM NADPH, 2 units of NADPH oxidase, 100 U of glutathione reductase, and 0.1 mg of dithiothreitol. After incubation at 37 °C for 10 min (100 μl), the reaction was stopped by addition of 1 ml of methanol. The oxidation products of testosterone were separated by HPLC and identified by their UV absorption and Rf values.
protein for rat and 0.2 mg of protein for human), 0.2 mM testosterone, and an NADPH generating system containing 0.8 mM NADP, 8.0 mM glucose 6-phosphate, 1 I.U. glucose 6-phosphate dehydrogenase and 6 mM magnesium chloride. When necessary, an ethyl acetate-extract of grapefruit juice was added to the reaction mixture. Vehicle (methanol 5 µl) was added to the control sample, instead of the extract. The reacting mixture was incubated for 15 min at 37 °C after preincubation for 2 min without the substrate, and then extracted with dichloromethane (1 ml) after the addition of an internal standard, 11α-hydroxyprogesterone. The organic residue after evaporation was dissolved in methanol and analyzed by a reversed-phase HPLC.

Chromatographic Conditions HPLC analysis was performed with Chemcosorb 5-ODS-H (5 µm, 150 × 6.0 mm i.d., Chemco Scientific Co., Ltd., Osaka, Japan) equipped with a precolumn packed with Nucleosil 120—5C18, (5 µm, 30 × 4.6 mm i.d., Chemco Scientific Co., Ltd., Osaka, Japan). A Jasco Model PU-980 HPLC pump was used for the analysis. Samples were introduced on the column via a Waters Model 712 Wisp autosampler. Metabolites were detected by the absorbance at 240 nm using a Jasco Model UV-970 variable wavelength UV-visible detector at room temperature. The mobile phase consisted of a multiple gradient of solvent A (water) and solvent B (methanol): 0 min, 60% (A); 10 min, 52%; 35 min, 40%; 40 min, 30%; 45 min, 30%; 50 min, 60% for analysis of testosterone metabolites and 0 min, 60% (A); 10 min, 52%; 35 min, 40%; 40 min, 30%; 45 min, 30%; 50 min, 10%; 75 min, 10%; 90 min, 60% for a fractionation of the components of grapefruit juice. The flow rate was set at 1 ml/min.

Known Flavonoid and Terpenoid Components Flavonoids (naringin and naringenin) and terpenoids (limonin and obacunone) were added to the reaction mixture as the respective methanolic or acetone solution. The final concentration used was in the range of 10—1000 µM for naringin, 10—500 µM for naringenin, 1—100 µM for limonin and obacunone.

Calculations The activity was shown as nmol formed per mg protein per min after calculation using a standard curve generated from the authentic standard. All values were expressed as the mean ± S.D. of triplicated determinations. The analysis of variance (ANOVA), followed by Scheffe’s multiple comparison of means, was used for statistical analyses.

RESULTS

Effect of Grapefruit Juice Extract on Testosterone Metabolism in Rat Liver Microsomes Testosterone is oxidized to several different regio- and stereoisomers in the livers of rats. Each reaction is catalyzed fairly selectively by specific forms of cytochrome P450. Microsomal 2α (16α)-, 6β (2β)-, and 16β-hydroxylations of this steroid are mediated by CYP2C11, CYP3A2 and CYP2B1/2 in the livers of normal male rats. In a preliminary experiment, original grapefruit juice and the ethyl acetate-extract showed nearly equivalent inhibition on microsomal testosterone hydroxylations (data not shown). Therefore, the ethyl acetate-extract of grapefruit juice was used to assess its effects on microsomal testosterone hydroxylation in the following study. In experiments to determine the inhibitory effect, the amounts of ethyl acetate-extract added to the reaction mixture was expressed as the corresponding volumes of original grapefruit juice in the reaction mixture; the reaction in 10% grapefruit juice equivalent is expressed as 0.1. As shown in Fig. 1A, microsomal testosterone hydroxylations at 6β-, 16α-, 2α- and 17-oxidation were decreased by the addition of grapefruit juice-extracts in a dose-dependent manner within the range of 0.0125—0.1 volumes of original grapefruit juice. Profiles of their decreases varied, dependent on the type of reaction. Microsomal 6β-hydroxylation was most profoundly affected, followed by 16α- and 2α-hydroxylation, and androstenedione formation, in a decreasing order. Additions of ca. 0.04, 0.05 and 0.1 volumes of grapefruit juice resulted in the 50% inhibition of metabolites (IC50), for 6β-, 16α (2α)-hydroxylations and 17-oxidation, respectively. Although grapefruit juice was acidic (pH 3–4), no clear change in pH was detected in the reaction mixture by the

Fig. 1. Inhibition of Microsomal Testosterone Metabolism in Livers of Rat (A) and Human (B) by Grapefruit Juice-Extract

6β-OHT, 16α-OHT and 2α-OHT indicate 6β-, 16α- and 2α-hydroxyltestosterone, respectively. To the reaction mixture (500 µl), amounts corresponding to 0.0125—0.1 volumes (6.25—50 µl) of grapefruit juice were added. Each value represents the mean ± S.D. of triplicate experiments. Asterisks denote statistically significant differences from each control value, *p < 0.05 and **p < 0.05.
addition of the ethyl acetate-extract (data not shown).

**Effect on Human Liver Microsomes** Components of grapefruit juice inhibited testosterone 6β-hydroxylation in human liver microsomes (Fig. 1B). To assess differences in the inhibitory effect among the commercially available juices, three different brands (A, B and C) were examined in this experiment. As shown in Fig. 1B, all three products showed inhibition of microsomal testosterone 6β-hydroxylation. No significant difference was detected among the three brands on the inhibitory effect, except for a point of 0.0125 between brands B and C.

**Addition of Naringin or Naringenin** Naringin is known to be a bitter principle in grapefruit. The inhibitory effects of naringin and its aglycone, naringenin, were tested in this assay system containing human liver microsomes (Fig. 2A). Although both chemicals decreased the formation of 6β-hydroxytestosterone in human liver microsomes, naringin showed less potency than naringenin (IC\textsubscript{50} > 1000 μM and ca. 50 μM, respectively).

Naringenin, however, was not detectable by HPLC in ethyl acetate-extracts of grapefruit juice, as described above, suggesting the involvement of a component other than naringenin in the inhibition of microsomal CYP3A activity (data not shown).

**Inhibition by Limonin and Obacunone** A triterpene-derived product, limonin, is contained in grapefruit. Obacunone is also a triterpene structurally-related to limonin. The possible contribution of these triterpenes was also examined. These chemicals reduced, but not as markedly, microsomal testosterone 6β-hydroxylation in human livers (Fig. 2B). Their IC\textsubscript{50} values for formation of the metabolite were ca. 100 μM and 65 μM, for limonin and obacunone, respectively.

**Screening of P450 Inhibitor by HPLC** To identify a specific P450 inhibitory component in grapefruit juices, the ethyl acetate-extract obtained from the juice of brand C was fractionated into six portions, every 15 min from 0 to 90 min, in a reversed-phase HPLC (Fig. 3A). Similar chromatograms were obtained among all brands used in this experiment. The inhibitory effect of each fraction was determined by microsomal testosterone 6β-hydroxylation in human livers. Clear decreases in the activity were detected in fractions 4 and 5, while the others had a minimal effect (Fig. 3B). Under this HPLC condition, authentic naringin is eluted in fraction 1, both authentic naringenin and limonin in fraction 2, and authentic obacunone in fraction 3. These fractions showed only weak effects. These results suggest that both fractions 4 and 5 contain unknown inhibitory components. Thus, both fractions were further examined after being subdivided into six portions, every 5 min between fractions 4 and 5 corresponding the retention time from 45 to 75 min, under the same analytical conditions. Clear inhibition of CYP3A4-mediated activity was detected in fractions 4-3 and 5-1 (Fig. 3C).

**DISCUSSION**

The present study clearly indicates the existence of specific inhibitory components for a CYP3A type of cytochrome P450 in grapefruit juice, which would be distinct from known components such as naringin, naringenin, limonin and obacunone.

Flavonoids in grapefruit juice have been suggested to be the major components involved in elevating the bioavailability of dihydropyridines. As grapefruit juice is known to be a rich source of naringin, quercetin, keampferol, apigenin and hesperetin, but these were scarcely or not at all detectable in orange juice. Several flavonoids have been shown to inhibit the CYP3A-catalyzed metabolism of dihydropyridines in human liver microsomes. As shown in Fig 2A, naringin and naringenin also inhibited microsomal testosterone 6β-hydroxylation in human livers. From the experiment shown in Fig. 1B, the IC\textsubscript{50} value of extracts of grapefruit juice (brand A) for the inhibition of 6β-hydroxytestosterone formation was determined to be ca. 0.05 volumes of grapefruit juice. Grapefruit juice is reported to contain naringin at the concentration of ca. 800 mg/L. In addition, more potent naringenin may be produced by the biotransformation of naringin. Thus, part of the inhibition observed may be explained by naringin. Nevertheless, coadministration of naringin with felodipine is reported to have no clear influence on the pharmacokinetics of the dihydropyridine. Thus, the
Fig. 3. HPLC Separation of Grapefruit Juice-Extract (A) and Their Inhibition of Microsomal Testosterone 6β-Hydroxylation in Human Liver (B and C)

Grapefruit juice-extract was separated as described in Materials and Methods (A). Fractionated extracts from experiments B (every 15 min from 0 to 90 min) and C (every 5 min from 45 to 75 min) were examined for their inhibitory effect on microsomal 6β-hydroxylation. To the reaction mixture (500 μl), amounts corresponding to 0.05 volumes (25 μl) of grapefruit juice were added. Each value represents the mean ± S.D. of triplicate experiments. Asterisks denote significant differences from each control value, *p < 0.05 and **p < 0.01.

role of flavonoids in vivo remains unclear. Limonin is an oxygenated tetranortriterpenoid that occurs generally in genus Citrus. This compound is found at concentration levels of 2–5 ppm in grapefruit as a bitter component. A structurally related product, obacunone, is also found in grapefruit juice. Both compounds inhibit microsomal CYP3A-dependent activity in human livers. The inhibitory effect of grapefruit juice-extract is, however, unlikely to be interpreted as caused by limonin and obacunone, since their amounts in grapefruit are too low to produce apparent inhibition. These observations suggest the presence of other inhibitory components in grapefruit juice. To identify CYP3A4-selective inhibitor(s) in grapefruit juice, ethyl acetate-extracts of grapefruit juice were fractionated by a reversed-phase HPLC. As shown in Fig. 3C, fractions 4-3 and 5-1, corresponding to retention times of 55–60 min and 60–65 min, showed clear inhibition on microsomal testosterone 6β-hydroxylation. Major peaks in both fractions were detected by the absorbance at both 240 nm and 313 nm. These peaks were not detected in orange juice. The UV spectra of these peaks resembled those of 7-propoxycoumarin and 5-methoxypsoralen, suggesting that the inhibitory components contain coumarin moieties in their molecules (data not shown). Preliminary experiments using LC/MS/MS (liquid chromatography/tandem mass spectrometry) further supported this idea. Exact identifications of these components are in progress.

In summary, the organic extracts of grapefruit juice are shown to contain at least two inhibitory components of human CYP3A. Although the exact structures remain unclear, these components are found to be distinct from flavonoids such as naringin and naringenin and limonin.

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