Differential UGT1A1 Induction by Chrysin in Primary Human Hepatocytes and HepG2 Cells

Cornelia M. Smith, Richard A. Graham, Wojciech L. Krol, Ivin S. Silver, Masahiko Negishi, Hongbing Wang, and Edward L. Lecluyse

Division of Drug Delivery andDisposition, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (C.M.S., R.A.G., H.W., E.L.L.); GlaxoSmithKline, Research Triangle Park, North Carolina (R.A.G., W.L.K., I.S.S.); and National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (M.N.)

Received June 9, 2005; accepted August 30, 2005

ABSTRACT

Chrysin, a dietary flavonoid, has been shown to markedly induce UGT1A1 expression and activity in HepG2 and Caco-2 cell lines; thus, it has been suggested to have clinical utility in the treatment of UGT1A1-mediated deficiencies, such as unconjugated hyperbilirubinemia or the prevention of 7-ethyl-10-hydroxycamptothecin (SN-38) toxicity. However, little is known about its induction potential in a more physiologically relevant model system, such as primary hepatocyte culture. In this study, induction of UGT1A1 expression (mRNA, protein, and activity) was investigated in primary human hepatocyte cultures after treatment with chrysin and other prototypical inducers. Endogenous nuclear receptor-mediated UGT1A1 induction was studied using transient transfection reporter assays in primary human hepatocytes and HepG2 cells. Results indicated that induction of UGT1A1 expression was minimal in human hepatocytes treated with chrysin compared with that in HepG2 cells (1.2- versus 11-fold, respectively). Subsequent experiments to determine whether the differential response was due to its metabolic stability revealed strikingly different elimination rate constants between the two cell systems (half-life of 13 min in human hepatocytes versus 122 min in HepG2 cell suspensions). Further study demonstrated that UGT1A1 mRNA expression could be induced in human hepatocyte cultures by either increasing the chrysin dosing frequency or by modulating chrysin metabolism, suggesting that the differential induction observed in hepatocytes and HepG2 cells was due to differences in the metabolic clearance of chrysin. In conclusion, this study suggests that the metabolic stability of chrysin likely would limit its ability to induce UGT1A1 in vivo.

The potential use of flavonoids as beneficial and therapeutic agents has gained widespread popularity over the years. The bioflavonoid chrysin, in particular, has been assessed by various investigators and proposed to be advantageous as an antioxidant, anticancer, anxiolytic, and anti-human immuno deficiency virus agent (Critchfield et al., 1996; Paladini et al., 1999; Chan et al., 2000; Galijatovic et al., 2001). Chrysin is naturally present in small amounts in honey, vegetables, fruits, and beverages (Uhl et al., 2003). Additionally, chrysin is sold as an androgen-boosting supplement due to its modulation of aromatase activity (Kellis and Vickery, 1984; Kao et al., 1998).

The mechanism(s) by which chrysin exerts these pharmacological effects has been explored, particularly its antioxidant and anticancer effects. These beneficial effects were deemed to result from the modulation of drug-metabolizing enzymes and those involved in the regulation of cellular oxidation processes. Through various in vivo and in vitro experiments, primarily conducted in rodents, chrysin was shown to induce or inhibit CYP1A activities (Tsyrlov et al., 1994; Moon et al., 1998; Breinholt et al., 1999), and chrysin metabolism was investigated in rat liver microsomes (Nielsen et al., 1998).
Because studies in humans were limited, other investigators examined disposition, metabolism, and induction potential of chrysin in clinical studies or immortalized human cell lines. The bioavailability of chrysin seemed to be very poor, and maximal plasma concentrations \((C_{\text{max}})\) were low (12–64 nM) after the administration of a single 400-mg oral dose in human volunteers (Walle et al., 2001). Furthermore, chrysin was found to be metabolized by UDP-glucuronosyltransferases (UGTs) and sulfotransferases in humans, Caco-2, and HepG2 cells (Galijatovic et al., 1999; Walle et al., 2001). More than one isooform belonging to the UGT1A subfamily was involved in the glucuronidation of chrysin. Chrysin was shown to be a substrate of UGT1A1, UGT1A6, and UGT1A9 with \(V_{\text{max}}\) values of 360, 157, and 1210 pmol/min/mg protein, respectively (Walle et al., 2000).

Additionally, chrysin was identified as an inducer of UGT in vitro because glucuronidation was greatly increased (14-fold over untreated) as determined by \(V_{\text{max}}/K_m\) in the chrysin-treated HepG2 cell homogenate (Walle et al., 2000). The increased in vitro clearance was due specifically to induction of UGT1A1 because chrysin did not induce UGT1A6, UGT1A9, or UGT1A4 (Walle et al., 2000). Moreover, UGT1A1-mediated bilirubin glucuronidation was increased 20-fold in HepG2 cells after treatment with 25 \(\mu\)M chrysin (Walle et al., 2000). These studies established that a phase II biotransformation reaction was the predominant clearance mechanism for the elimination of chrysin and depicted that chrysin might possess a great potential to induce UGT1A1 in humans.

Typically, the UGT enzymes have not been found to be induced to the same extent as cytochrome P450 enzymes in parallel studies. Three- to 4-fold induction of UGT1A1, at best, has been observed in our laboratory and those of others using prototypical inducing agents in primary human hepatocytes (HH) (Sutherland et al., 1993; Brierley et al., 1996; Ritter et al., 1999; Soars et al., 2004; Smith et al., 2005). As such, the large UGT1A1 induction response observed in immortalized cell lines after chrysin exposure seems rather atypical. Immortalized cell lines may not be the best model system for assessment of enzyme induction, because important transcription factors, cofactors, transporters, and some drug-metabolizing enzymes may be decreased or absent compared with other more physiologically relevant models.

As part of this study, a more systematic evaluation of the induction of UGT1A1 by chrysin was performed using HH, which are more representative of in vivo conditions (LeCuyse, 2001). Because the expression of drug-metabolizing enzymes, transporters, and transcriptional factors is greater in HH than in immortalized hepatoma cell lines, it was hypothesized that chrysin would not induce UGT1A1 expression significantly in HH due to extensive metabolism. Experiments were conducted in both HH and HepG2 cell cultures to determine the induction of UGT1A1 mRNA, immunoreactive protein, and activity after treatment with chrysin over a range of concentrations. Because the \(UGT1A1\) gene contains functional elements that are responsive to constitutive androstane receptor (CAR), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AhR) agonists (Sugatani et al., 2001; Xie et al., 2003; Yueh et al., 2003), transient transfection assays were conducted in both HH and HepG2 cells to better understand the mechanism of its regulation by chrysin. Metabolic stability studies were undertaken in suspensions of freshly isolated and cryopreserved human hepatocytes and HepG2 cells to determine the differences in chrysin clearance. Borneol, an inhibitor of glucuronidation (Watkins and Klaassen, 1983), was used to aid further the characterization of the cell-dependent chrysin induction response.

**Materials and Methods**

**Chemicals.** Chrysin (CHRY), 3-methylcholanthrene (3-MC), phenobarbital (PB), dimethyl sulfoxide (DMSO), perchloric acid, \(\beta\)-estradiol (E2), \(\beta\)-estradiol-3-glucuronide (E3G), dexamethasone, dexamethasone, dexamethasone, dexamethasone, and bovine serum albumin (ITS) \(^*\) were obtained from BD Biosciences Discovery Labware (Bedford, MA). The human hepatoma cell line HepG2 was purchased from American Type Culture Collection (Manassas, VA). Collagenase type IV was from Sigma-Aldrich, TRIZol reagent was from Invitrogen. A polyclonal antibody against human UGT1A1 was purchased from BD Biosciences Discovery Labware, and CYP1A2 antibody was from Chemicon International (Temecula, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Zymed Laboratories (South San Francisco, CA). CellPhect transfection kit was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The dual-luciferase reporter assay System was from Promega (Madison, WI). All other biological reagents were purchased from commercial suppliers, and they were either of American Chemical Society or molecular biology grade.

**Isolation and Culturing of Human Hepatocytes.** Human hepatocytes were obtained either by isolation from liver tissues obtained from University of North Carolina Hospitals or from commercial sources (CellzDirect, Tucson, AZ; ADMET Technologies, Research Triangle Park, NC). Hepatocyte isolations performed in the investigators’ laboratory were conducted as described by LeCuyse et al. (2005). Hepatocytes were cultured on Biocoat plates (BD Biosciences, Bedford, MA). The human hepatoma cell line HepG2 was purchased from American Type Culture Collection (South San Francisco, CA). The dual-luciferase reporter assay System was from Promega (Madison, WI). All other biological reagents were purchased from commercial suppliers, and they were either of American Chemical Society or molecular biology grade.

**Induction Studies.** Cultures of hepatocytes were maintained in modified Chee’s medium supplemented with ITS* and 0.1 \(\mu\)M dexamethasone for 36 to 48 h before exposure to inducers. HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen). Hepatocytes or HepG2 cells were treated with vehicle (0.1% DMSO) alone, chrysin (1–50 \(\mu\)M), or 3-MC (1 or 5 \(\mu\)M) for three consecutive days for protein expression or catalytic activity assessment. Levels of UGT1A1 mRNA were assessed following a 24- to 72-h treatment period. In a separate study, HH were exposed to either a single dose of 10 \(\mu\)M chrysin or repeat doses of 10 \(\mu\)M chrysin every 2 h for 12 h. Control (CTL) groups included a single dose of 0.1% DMSO and multiple dosing of 0.1% DMSO every 2 h for 12 h. At the end of each treatment period, cells were harvested in TRIZol reagent or homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM KCl, and 2 mM EDTA) (LeCuyse et al., 2005). Protein concentrations were determined following the manufacturer’s instructions using a commercially available kit (BCA protein assay; Pierce Chemical, Rockford, IL).
The human CYP2B6 luciferase reporter plasmids containing the phenobarbital-responsive enhancer module (PBREM) alone or both the PBREM and the distal xenobiotic responsive enhancer module (PBREM-XREM) were generated previously (Sueyoshi et al., 1999; Wang et al., 2003). The pSG5-hPXR expression vector was provided generously from Dr. Steven Kliewer (GlaxoSmithKline, Research Triangle Park, NC).

**Transient Transfection Assays.** HepG2 cells were seeded in 24-well plates at 8 × 10^4 cells/well in phenol red-free DMEM-Ham's F-12 supplemented with 10% charcoal-stripped fetal calf serum the day before transfection. HH were seeded also in 24-well plates and cultured in Williams' medium E (Sigma-Aldrich). In HepG2 cells, 100 ng of reporter plasmid and 10 ng of pRL-TK vector in DMEM-Ham's F-12 were transfected using CellPhect transfection reagent (QIAGEN, Valencia, CA). Subsequently, transfection complexes were removed and cells (HH and HepG2) were dosed with vehicle or test compound for 24 h. Using the dual-luciferase reporter assay system (Promega), promoter activities were determined from vehicle or test compound for 24 h. Using the dual-luciferase reporter assay protocol (QIAGEN, Valencia, CA). Subsequently, transfection complexes were removed and cells (HH and HepG2) were dosed with vehicle or test compound for 24 h. Using the dual-luciferase reporter assay system (Promega), promoter activities were determined from vehicle or test compound for 24 h.

**Metabolic Stability Studies and Liquid Chromatography-Mass Spectrometry Analysis.** Metabolism studies were conducted in freshly isolated hepatocytes, cryopreserved hepatocytes (L-OQD; In Vitro Technologies, Baltimore, MD), and HepG2 cells. Cell suspensions consisting of 0.5 × 10^6 viable cells/incubation were diluted in serum-free DMEM supplemented with ITS (100X), 0.1 μM dexamethasone, and penicillin-streptomycin. Cells were incubated in duplicate under standard culture conditions with chrysin at a final concentration of 0.5 μM. Time points used were 0, 15, 30, 60, 90, and 120 min, and reactions were terminated with 1% acetic acid in 80:20 acetonitrile/methanol. Peak areas of parent compound and metabolites were determined using a Cohesive Technologies (Franklin, MA) Aria TX-2 HTLC system interfaced to a API mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray source.

**Determination of UGT1A1-Specific Activities and HPLC Analysis.** UGT1A1 catalytic activities were determined by the quantification of E3G formation in human hepatocyte monolayers immediately following the 3-day treatment period. Dosing media were removed, and hepatocytes were equilibrated for 5 to 10 min in Krebs-Henseleit buffer (Sigma-Aldrich) under typical cell culture incubation conditions. After the washout period, hepatocyte monolayers were exposed to 100 μM E_2 diluted in Krebs-Henseleit buffer for 30 min and then transferred to −70°C to stop the metabolism of E_2. At a later time, media and cells were thawed and collected for analysis and protein determination, respectively. Perchloric acid (30 μl; 6%) and dextromethorphan (20 μl; 5 ng/μl used as internal standard) were added to 250 μl of media (Alkhafry and Frye, 2002). Samples run in triplicate were then centrifuged, and 50 μl of supernatant was injected for HPLC analysis.

E3G formation was determined via reverse-phase high-performance liquid chromatography with fluorescence detection as reported by Alkhafry and Frye (2002) and described previously by Smith et al. (2005). A standard curve that ranged from 100 to 10000 ng/ml was constructed from E3G standards diluted in 250 μl of Krebs-Henseleit buffer was used to calculate the activities of the unknown samples.

**Immunoblotting and Densitometric Analyses.** Homogenates (20–30 μg) were prepared in SDS sample loading buffer containing β-mercaptoethanol and resolved on SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes. Membranes were blocked and washed in 1X casein solution before incubation with primary antibodies against UGT1A1 or CYP1A2 diluted 1:1000 and 1:5000, respectively. Blots were washed and incubated with horseradish peroxidase goat anti-rabbit IgG antibody diluted 1:10000 in 1X casein solution. Blots were developed using ECL Western blotting detection reagent (GE Healthcare) and visualized with a Versa-Doc 1000 imaging system (Bio-Rad, Hercules, CA). Densitometry to determine the relative amounts of protein was performed using NIH Image software.

**Determination of UGT1A1 mRNA Expression by Real-Time PCR Analysis.** Total RNA was isolated from HH or HepG2 cells using RNeasy mini kit (QIAGEN). Total RNA from each treatment group was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen). The primers and TaqMan probe specific for human UGT1A1 were designed using Primer Express 2.0 software (Applied Biosystems) and made up the following sequences: forward primer, 5'-GGGCCATCATGCCCATAT-3'; reverse primer, 5'-TTGAAATTTCCGGGATATGGATT-3'; and probe, 6FAM-TTTTTGTTGTTGGGATACGTCCTCAG-TAMRA. For an internal control, β-actin mRNA was measured using predeveloped Taqman primer and probe mixture from Applied Biosystems (catalog no. 4310881E). The mRNA quantification of UGT1A1 was normalized to β-actin mRNA and expressed as fold induction over CTL. Amplification and detection were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems).

**Statistics.** Catalytic activities, luciferase activities, and mRNA levels are presented as a mean of triplicate determinations ± S.D. unless otherwise noted. Statistical comparisons were made where appropriate using a one-way analysis of variance, Dunnett's method, to compare treatment groups with control groups. The criterion of significance was set at p = 0.05, and tests were performed using SigmaStat version 2.03 software (SPSS Inc., Chicago, IL).

**Table 1: Demographics of Human Liver Donors in Hepatocyte Culture**

<table>
<thead>
<tr>
<th>Liver Donor Identification</th>
<th>Age</th>
<th>Gender</th>
<th>Race</th>
<th>Experimental Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-105</td>
<td>62</td>
<td>Female</td>
<td>Caucasian</td>
<td>CHRY concentration-response profiles</td>
</tr>
<tr>
<td>L-001</td>
<td>63</td>
<td>Male</td>
<td>Caucasian</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>L-004</td>
<td>18</td>
<td>Male</td>
<td>African-American</td>
<td>Metabolism studies</td>
</tr>
<tr>
<td>L-004B</td>
<td>3</td>
<td>Female</td>
<td>Caucasian</td>
<td>Gene reporter assays</td>
</tr>
<tr>
<td>L-OQD</td>
<td>51</td>
<td>Male</td>
<td>Caucasian</td>
<td>Metabolism studies</td>
</tr>
<tr>
<td>L-002</td>
<td>50</td>
<td>Male</td>
<td>N.A.</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>L-006</td>
<td>34</td>
<td>Female</td>
<td>Caucasian</td>
<td>Gene reporter assays, CHRY multiple dosing</td>
</tr>
<tr>
<td>L-151</td>
<td>78</td>
<td>Female</td>
<td>Caucasian</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>L-117</td>
<td>58</td>
<td>Female</td>
<td>Caucasian</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>L-214</td>
<td>61</td>
<td>Female</td>
<td>Caucasian</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>L-230</td>
<td>74</td>
<td>Female</td>
<td>Caucasian</td>
<td>mRNA expression</td>
</tr>
<tr>
<td>L-169</td>
<td>37</td>
<td>Female</td>
<td>African-American</td>
<td>UGT inhibition by BORN</td>
</tr>
</tbody>
</table>

N.A., not available.

*Donor information not available.
Results

UGT1A1 Induction by Chrysin in HepG2 Cells. The induction of UGT1A1 expression was examined in the human hepatoma cell line HepG2 using 3-MC as a prototypical AhR activator and UGT1A1 inducer (Li et al., 1998; Walle and Walle, 2002; Zhang et al., 2003). The compounds 3-MC (1 μM) and chrysin (10 μM) induced UGT1A1 mRNA levels 15- and 11-fold, respectively, above 0.1% DMSO-treated cells (CTL) (Fig. 1A). Moreover, a substantial increase in UGT1A1 mRNA expression was observed with the 25 μM chrysin treatment. Additional experiments were conducted using reporter constructs containing the UGT1A1-gtPBREM element or the CYP2B6 PBREM element in the presence or absence of exogenously expressed human PXR to assess the mechanism(s) of UGT1A1 regulation by chrysin. The functional elements of both the UGT1A1-gtPBREM [PXR response element, CAR response element, and AhR response element (AhRE)] and CYP2B6 promoter [nuclear receptor binding sites (NR1, NR2, and NR3)] are illustrated in Fig. 1B. These genes responded differentially to the AhR agonists chrysin and 3-MC. Chrysin did not activate PXR, behaving in a manner similar to that of 3-MC, because CYP2B6-PBREM-XREM reporter activation remained unaffected compared with CTL (Fig. 1C). However, CYP2B6 reporter activity was induced by PB in the presence of PXR (Fig. 1C). In contrast, both 3-MC and chrysin increased gtPBREM reporter activity but not CYP2B6-PBREM reporter activity by endogenously expressed AhR in HepG2 cells (Fig. 1D).

UGT1A1 Response to Chrysin in HH. HH were treated with 0.1% DMSO, 3-MC, or various concentrations of chrysin ranging from 1 to 50 μM to obtain a concentration-response profile. UGT1A1 and CYP1A2 protein levels were induced 2- and 13-fold, respectively, by 5 μM 3-MC (Fig. 2, A–D). Statistically significant induction of UGT1A1 activity was observed in hepatocyte cultures treated with 3-MC (p < 0.05) (Fig. 2E). However, UGT1A1 activities and protein levels were not increased by chrysin treatment at multiple concentrations. To illustrate that the effect was not limited to UGT1A1 gene expression, a similar result was obtained when the protein levels of CYP1A2 were examined in hepatocytes from the same donor (Fig. 2, B and D).

Additional experiments were conducted in HH to assess UGT1A1 reporter luciferase activity and UGT1A1 mRNA expression. HH isolated from several donors (n = 4) were transfected with the gtPBREM reporter construct and treated with chrysin or 3-MC. The majority of the hepatocyte preparations responded to 3-MC with significant increases (p < 0.05) in UGT1A1 reporter activity (Fig. 3A). However, none of the hepatocyte preparations responded to chrysin significantly. A similar pattern was observed when the
mRNA expression levels were investigated in HH treated with chrysin (n = 5 donors). \textit{UGT1A1} mRNA levels were induced by 3-MC 4- to 10-fold over CTL levels, whereas chrysin did not elicit further increases in \textit{UGT1A1} mRNA expression beyond CTL levels (Fig. 3B).

**Metabolic Stability of Chrysin in HepG2 and Hepatocytes.** To determine whether the lack of response in HH after exposure to chrysin might be due in part to metabolic clearance, HepG2 cells and both cryopreserved and freshly isolated human hepatocyte suspensions were used to compare the in vitro half-life of chrysin. A nearly 10-fold difference in elimination rate constants ($k$) was observed between the cell systems, 0.053 and 0.0057 min$^{-1}$ in HH and HepG2, respectively (Fig. 4). Using these constants, a half-life ($t_{1/2}$) of 13 versus 122 min was determined in HH and HepG2 cells, respectively. There was virtually no difference in the half-life determined from freshly isolated and cryopreserved hepatocytes (13.1 and 13.2 min, respectively). Moreover, only 1% of the parent compound was present at 90 min in the HH, whereas 65% of chrysin remained in HepG2 cells after 90 min. In fact, a considerable percentage of chrysin (50%) still remained in HepG2 cells at 120 min. When the metabolism of parent compound was examined in primary cultures of hepatocytes, the concentrations also were decreased (data not shown).

**Glucuronide and Sulfate Metabolite Formation in HepG2 and Hepatocytes.** Glucuronide and sulfate conjugates were detected in both HepG2 and human hepatocyte cell suspensions (Fig. 5). Standards were not available for chrysin-glucuronide (CHRY-Gluc) and chrysin-sulfate (CHRY-Sulf); therefore, results are expressed as a percentage of total peak area of all analytes for each particular time point. A striking difference between CHRY-Gluc and CHRY-Sulf formation was observed in HepG2 cells. The percentage of sulfate metabolite was much greater than that of glucuronide (Fig. 5A). The sulfate metabolite increased over time and seemed to level off after the 60-min time period. The majority of chrysin was recovered as CHRY-Sulf at 120 min (74%) in HepG2. In contrast, CHRY-Gluc and CHRY-Sulf percentages were not very different from each other in hepatocytes, and they seemed to level off after 30 min (Fig. 5B). The percentages of CHRY-Gluc and CHRY-Sulf formed were 44 and 54, respectively, at the 120-min time point. Direct comparison of the conjugates formed in HepG2 and hepatocytes.
cytes shows that the sulfate metabolite occurred readily in both cell systems. However, the glucuronide metabolite was present in nearly equal amounts as the sulfate conjugate in HH suspensions, whereas the amount of glucuronide in HepG2 cells was negligible.

Effects of Multiple Dosing of Chrysin in HH. Because extensive metabolism of chrysin was observed in HH, it was postulated that this might be the reason why chrysin was unable to exert its inducing effects on UGT1A1 expression in HH. An experiment was designed to maintain a more constant concentration of chrysin in the culture medium by increasing the frequency of dosing. HH were subjected to multiple dosing (MULTI) with 10 μM Chrysin or 0.1% DMSO every 2 h for a period of 12 h. Parallel cultures were treated for 12 h with a single dose of 0.1% DMSO, 5 μM 3-MC, or 10 μM chrysin, referred to as standard dosing (STD). Statistically significant increases in UGT1A1 mRNA were observed with both the chrysin MULTI treatment and the 3-MC treatment (Fig. 6A). In contrast, the chrysin STD dose did not elicit a significant increase in UGT1A1 mRNA expression.

Inhibition of Glucuronidation in HH. Results from the metabolic stability studies and multiple-dosing experiments suggested that rapid metabolism of chrysin may be a plausible explanation for the differential induction ob-

---

Fig. 3. UGT1A1 reporter activities and expression in HH. A, hepatocytes (n = 4 donors) were cotransfected with gtPBREM and pRL-TK-promoter constructs, and luciferase activities were determined after a 24-h treatment with 0.1% DMSO, 1 μM 3-MC, or 10 μM CHRY. B, HH (n = 5 donors) were treated with 0.1% DMSO, 5 μM 3-MC, or 10 μM CHRY, and UGT1A1 mRNA levels were determined by real-time PCR (TaqMan) normalized to β-actin mRNA, and expressed as fold induction over CTL. Results are presented as means ± S.D. of triplicate measurements, and statistical significance is denoted with an asterisk (p < 0.05).

Fig. 4. Metabolic stability of CHRY in HepG2 cells and human hepatocytes. The metabolic stability of CHRY was determined in suspensions of HepG2 cells, freshly isolated hepatocytes, and cryopreserved hepatocytes by monitoring the disappearance of CHRY over time (0, 15, 30, 60, 90, and 120 min). The half-life of CHRY was estimated under the assumption that the disappearance followed a first-order linear kinetic process. Data points represent the average of two determinations with bars indicating the range of variation.

Fig. 5. Metabolites of CHRY in HepG2 Cells and HH. Suspensions of HepG2 cells or human hepatocytes were incubated with 0.5 μM CHRY over time (0, 15, 30, 60, 90, and 120 min). Metabolites were observed in HepG2 (A) and HH (B) where glucuronide and sulfate conjugates of CHRY are represented by CHRY-Gluc and CHRY-Sulf, respectively. All analytes were expressed as a percentage of total peak areas at each time point, and the parent profile is shown as a reference. Data points represent the average of two determinations with bars indicating the range of variation.
Chrysin greatly increased UGT1A1 expression in immortalized cell lines, such as Caco-2 and HepG2 cells, in previous reports (Galijatovic et al., 2000, 2001; Walle et al., 2000). Our studies confirmed the large induction observed in HepG2 cells. However, only a few reports have focused on the mechanism by which chrysin induces drug-metabolizing enzymes. Zhang et al. (2003) found that the induction of CYP1A1 reporter activity by chrysin was mediated through AhR activation and subsequent binding to dioxin-responsive elements present in the gene in HepG2 cells. In a separate study, Sugatani et al. (2004) found that the UGT1A1 response to chrysin was alleviated the most when the AhRE within the gtPBREM was mutated in transactivation experiments conducted in HepG2 cells. UGT1A1 and CYP2B6 gene reporter activation by chrysin and 3-MC was compared in HepG2 cells in the present study. CYP2B6 was not responsive to chrysin, but it was responsive to PB, a PXR and CAR activator, when the PBREM was cotransfected with PXR. UGT1A1 was responsive to chrysin and 3-MC in a PXR-independent manner. Although the expression of some nuclear receptors such as PXR and CAR is nonexistent or subdued in HepG2 cells, the AhR is endogenously expressed in these cells (Roberts et al., 1990; Lemaire et al., 2004; Rencurel et al., 2005). These results suggested that chrysin regulation is AhR-mediated because a functional AhRE is present in the UGT1A1 promoter, whereas it is not in the CYP2B6 promoter (Fig. 1).

The effects of chrysin on UGT1A1 expression were evaluated in sandwich-cultured primary human hepatocytes. Although there are limitations associated with this model system, HH retain more of their cellular characteristics than do immortalized cell lines, and they are an ideal and established in vitro model to assess drug-metabolizing enzyme induction (leCluyse, 2001). To our knowledge, this is the first study to examine the induction potential of chrysin on UGT1A1 expression in HH. Our results showed that its effects were minimal in HH, thus leading to the discovery that chrysin exhibits differential effects on UGT1A1 gene expression in HH and HepG2 cells. The reasons for these differential results were postulated and investigated.

The metabolism of chrysin had been investigated previously in the human Caco-2 and HepG2 immortalized cell lines. In those studies, chrysin was found to undergo glucuronidation and sulfation, which was identified as the cause of the observed low bioavailability in a clinical study (galijatovic et al., 1999; Walle et al., 2001). Upon examination of the rate of metabolism, investigators found that 50% chrysin was metabolized in 6 h, and <10% parent compound remained after a 24-h incubation period (Galijatovic et al., 1999). The metabolic stability of chrysin in the HH and HepG2 cell systems was compared in the present study. Considerable differences (~10-fold) were found in the rates of metabolism of chrysin between HH and HepG2, resulting in strikingly different in vitro half-lives. Moreover, it seemed that little glucuronide was formed in HepG2 cells in our studies, suggesting that this pathway may not be relevant in these cells for the clearance of chrysin.

It was postulated that the differences in UGT1A1 induction might be partially explained by rapid metabolism of chrysin in HH compared with HepG2 cells, thereby resulting in a lower amount of chrysin available for nuclear receptor-
mediated transcriptional activation. To test this possibility, two types of experiments were conducted. The first examined the effects of increasing the dosing frequency with the intention of maintaining the concentration of chrysin in the medium at a more constant level. The second experiment examined chrysin induction in the absence or presence of BORN. Several studies have used BORN as an inhibitor of glucuronidation based on its ability to deplete the cofactor UDP-glucuronic acid needed to facilitate biotransformation reactions by UGTs, and the concentrations used were similar to the ones used in this study (Watkins and Klaassen, 1983; Porubek et al., 1989; Kretz-Rommel and Boelsterli, 1993; Grillo et al., 2003). The combined results of this study suggested that the metabolism of chrysin could be a major factor in its capacity to induce UGT1A1. When the dosing frequency of chrysin was increased, which presumably resulted in increased cellular concentrations of chrysin and subsequent activation of the AhR, increased levels of UGT1A1 mRNA were observed compared with those in HH exposed to a single dose. It was observed also that UGT1A1 mRNA levels increased as the concentration of the UGT inhibitor BORN increased in the presence of a single concentration of chrysin (10 μM). It would not necessarily be expected that the induction of UGT1A1 expression by chrysin in the presence of a glucuronide inhibitor would reach its full induction potential because chrysin is metabolized also via sulfation or, if not, other pathways yet to be identified in humans. Under the conditions used in this study, both glucuronidation and sulfation seemed to contribute equally to chrysin metabolism in hepatocyte studies (Fig. 5B). As such, additional inhibitors could be used to block alternate metabolic pathways of chrysin. For example, triclosan, an antibacterial agent, was recently identified as a potent inhibitor of the sulfation and glucuronidation (to a minor extent) of several compounds in human liver fractions (Wang et al., 2004). Triclosan, in addition to BORN, could be used to delineate the contribution of glucuronidation versus sulfation in the metabolism of chrysin in human hepatocyte culture.

Another reason for the apparent differences in induction potential in HH and HepG2 cells may be that the response of UGT1A1 to chrysin in HepG2 cells is artificially exaggerated because of its extraordinarily low basal expression in these cells. In contrast, the basal expression of UGT1A1 in whole liver and human hepatocytes is relatively high, especially compared with that of HepG2 cells, which may inherently diminish the pharmacological response observed in the presence of an inducer (Smith et al., 2005). In addition, chrysin is a substrate for several UGT isozymes, and UGT1A9 was shown to be an important contributor to its glucuronidation by Walle et al. (2000). The expression of other UGTs, including UGT1A9, could also be low in HepG2 cells, in which case the effects of their contribution would not be observed or be greatly diminished. However, in HH where all liver-specific isozymes are endogenously expressed, chrysin has a much greater chance of undergoing metabolism as would be expected in vivo.

Based solely on results observed in both HepG2 and Caco-2 immortalized cell lines, which have shown that chrysin is a proficient modulator of UGT1A1 expression, it has been proposed that chrysin may have practical applicability to physiological conditions related to UGT1A1 deficiencies, such as unconjugated hyperbilirubinemia and SN-38-mediated gastrointestinal toxicity (Galijatovic et al., 2001; Walle and Walle, 2002; Sugatani et al., 2004). Our results indicate that use of chrysin to replace existing drug therapies (e.g., PB therapy) or in metabolic diseases prevalent in UGT1A1-deficient individuals may not be physiologically feasible in vivo because it undergoes extensive metabolism. Upon biotransformation, only a minute percentage of chrysin is available in HH, thus decreasing its chance to induce UGT1A1 expression. This hypothesis is supported by the results obtained in a recent clinical study that found Cmax values of chrysin to range from 12 to 64 nM in human volunteers administered a single oral 400-mg dose (Walle et al., 2001). These clinical results combined with our findings lead to the prediction that marked induction of UGT1A1 will not occur in vivo, except under conditions where UGT or SULT enzyme activity or expression were severely impaired.

In conclusion, chrysin differentially induced UGT1A1 in HH and HepG2 cells. Although chrysin elicited a large effect on UGT1A1 expression in HepG2, minimal effects were observed in HH. The observation that UGT1A1 induction was greater upon inhibition of chrysin glucuronidation or multiple dosing of chrysin to the cells suggests that the induction is influenced by its metabolic stability in culture. Notably, additional flavonoids have been investigated for their UGT1A1 induction potential, and some (acacetin, apigenin, luteolin, and diosmetin) have been identified as inducers of UGT1A1 activity in HepG2 cells (Walle and Walle, 2002). Because of the contrasting results observed in these studies, it is beneficial to include HH in studies designed to examine the effects of compounds structurally related to chrysin on liver gene expression. Extrapolation to the in vivo situation should be made with caution when using immortalized cell lines to assess endogenous enzyme induction because the cellular composition of a particular cell system could confound the results and lead to incorrect predictions of the in vivo situation. The results of these studies further demonstrate that primary hepatocyte culture represents a more relevant model system for studying the effects of metabolically labile compounds.

Acknowledgments

Human liver tissue was procured with the assistance of Drs. Benjamin Calvo and Kevin Behrens (University of North Carolina at Chapel Hill Hospitals) and Lynn Johnson and Evangeline Reynolds (Lineberger Comprehensive Cancer Center Tissue Procurement Program, University of North Carolina). CellzDirect and ADMET Technologies provided human hepatocytes for some of the studies. We thank Dr. Philip C. Smith for helpful suggestions related to the chrysin glucuronidation inhibition experiments. We also thank Drs. Elaine M. Leslie, Kim L. R. Brouwer, and Stephanie R. Faucette for suggestions and careful critique of this manuscript.

References


Critchfield JW, Butera ST, and Folks TM (1998) Inhibition of HIV activation in...


Address correspondence to: Dr. Hongbing Wang, Division of Drug Delivery and Disposition, School of Pharmacy, CB 7360, Kerr Hall, Room 2319, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360. E-mail: hongbing_wang@unc.edu