Induction of UDP-Glucuronosyl-Transferase by the Flavonoids Chrysin and Quercetin in Caco-2 Cells

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Purpose. Dietary flavonoids have been reported to be potent inhibitors of drug metabolizing enzymes. In the present study we examined the inducing effect of three of these compounds, chrysin, quercetin and genistein, on UDP-glucuronosyltransferase (UGT) in the human intestinal cell line Caco-2.

Methods. The induction of UGT by flavonoid pretreatment was studied both in the intact cells and cell homogenates, measured as the glucuronidation of chrysin, and by immunoblot analysis of the UGT 1A protein.

Results. Exposure of Caco-2 cells to 50 μM chrysin resulted in a 3.8-fold increase in chrysin glucuronidation in intact cells ($p < 0.0001$) with a 38% decrease in sulfation ($p < 0.01$). In the cell homogenate the induction was much larger, 14-fold. The induction was slow to develop with maximum induction after 3–4 days. Interestingly, the isoflavonoid genistein was without effect. Immunoblot analysis of Caco-2 cell microsomes with a UGT1A subfamily-selective antibody showed a markedly increased band at about 59 kDa, consistent with induction of one or more UGT1A isoforms. A 5-week exposure of Caco-2 cells to low concentrations (10 μM) of chrysin or quercetin also showed markedly increased glucuronidation activity.

Conclusions. Diet-mediated induction of intestinal UGT may be important for the bioavailability of carcinogens and other toxic chemicals as well as therapeutic drugs.

KEY WORDS: flavonoids; chrysin; quercetin; induction; glucuronidation; UDP-glucuronosyltransferase; Caco-2 cells.

INTRODUCTION

As emphasized during the continuing development of the Caco-2 cell line as a model for human intestinal absorption, these cells in culture morphologically resemble small intestine absorptive cells with many of its typical enzymes and transporters (1–4). Although Caco-2 cells in the past have not been considered to express high levels of drug metabolizing enzymes (5,6), conjugation reactions have previously been described. This includes sulfation of dopamine and *p*-nitrophenol (7) and L-α-methyldopa (8). The model substrate *p*-nitrophenol has also been shown to be glucuronidated by Caco-2 cells (5,9), as has L-α-methyldopa (8).

In several recent studies (4,10) we used Caco-2 cells to characterize the human intestinal absorption of the dietary flavonoids. For one of these natural products, chrysin, the limiting factor was not membrane penetration but rather metabolism through glucuronidation and sulfation (4). The catalytic efficiency of both conjugation pathways for chrysin was very high, similar in magnitude to fresh rat hepatocytes (11).

In the process of these studies, we found evidence of induction of UDP-glucuronosyltransferase (UGT) by flavonoids in the Caco-2 cells. The only previous report of a flavonoid inducing a UGT activity in a human cell involved the isoflavonoid biochanin A, which increased the glucuronidation of testosterone in an androgen-responsive prostate cancer cell line (12). However, studies in the rat had previously provided evidence that flavonoids may be inducers of UGT (13,14).

This report describes for the first time the induction of UGT by chrysin and the ubiquitous dietary flavonoid quercetin (Fig. 1) in Caco-2 cells.

MATERIALS AND METHODS

Materials

Chrysin, quercetin, genistein, uridine 5′-diphosphoglucuronic acid (UDPGA) and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, Mo). Trifluoroacetic acid was of spectrophotometric grade from Aldrich Chemical Co. (Milwaukee, WI). Hanks' balanced salts solution (HBSS) was obtained from Cellgro, Mediatech, Fisher Scientific (Pittsburgh, PA). Recombinant human UGT1A6 and the corresponding Western blotting kit were purchased from Gentest Corp. (Woburn, MA). Polyclonal anti-human UGT1A antibodies, raised against 15 amino acids in the common C-terminal end of the UGT1A proteins, were a generous gift from Gentest. Secondary antibody, HRP-conjugated goat anti-rabbit IgG, was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). SuperSignal Chemiluminescent Substrate was from Pierce (Rockford, IL). Electrophoresis and blotting supplies and prestained molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA).

Caco-2 Cell Culture

The human colon adenocarcinoma cell line Caco-2 was obtained from American Type Culture Collection (Rockville, MD). The Caco-2 cells were cultured in Eagle's Minimum Essential Medium with Earle's salts and L-glutamine (Cellgro, Mediatech, Fisher Scientific, Pittsburgh, PA), supplemented with 1% non-essential amino acids (Cellgro), 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO) and penicillin/streptomycin (Sigma Chemical Co.). Cells were grown in humidified air with 5% CO_2 and subcultured in 6-well plates for incubation experiments and 100 mm petri dishes for homogenate and microsomal preparation. The cells were used at passage 35–75.

Chrysin Metabolism by Pretreated and Control Caco-2 Cells

Four days after seeding Caco-2 cells in 6-wells, pretreatment with 50 μM chrysin or 50 μM genistein in complete cell culture medium was started. The medium was changed every 24 hr. Chrysin was dissolved in ethanol:DMSO (80:20, v/v) at

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HBSS, Hanks' balanced salt solution; UDPGA, uridine 5′-diphosphoglucuronic acid.

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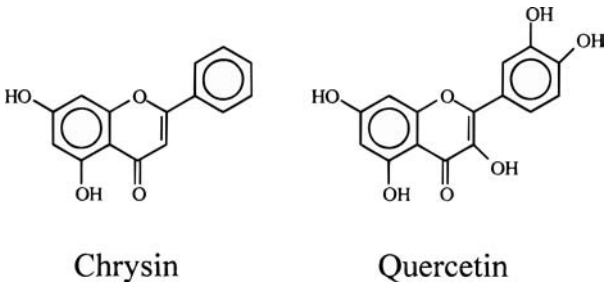

Fig. 1. Chemical structures of chrysin and quercetin.

a final concentration not exceeding 0.5%. Control cells were treated with the same volume of solvent. After 4 days of pretreatment, 50 μM chrysin was added to both chrysin-treated, genistein-treated and control cells. The medium was harvested 24 hr later for analysis of chrysin and metabolites. Conjugated metabolites and unmetabolized chrysin were separated from the cellular medium using Oasis HLB 3cc extraction cartridges (Waters, Milford, MA). The methanol eluates were taken to dryness and the samples were reconstituted in 250 μl mobile phase and analyzed by reversed phase HPLC, using a Symmetry C18 column (Waters) with 55% methanol and 0.3% trifluoroacetic acid as the mobile phase at 0.9 ml/min with 268 nm UV detection (11). The cells were digested with 0.5 M NaOH and analyzed for protein content (15).

In another set of experiments, Caco-2 cells in 6-wells were pretreated with 50 μM chrysin for 1, 2, 3 or 4 days. The medium was analyzed for chrysin metabolites 24 hr after the last addition of chrysin (8 days after plating). The effect of flavonoid pretreatment concentration on the glucuronidation was investigated using 0, 5, 10, 25 and 50 μM chrysin for 4 days.

Preparation of Caco-2 Cell Homogenate and Microsomes

Caco-2 cells were plated in 100-mm petri dishes. On day 6 the cells were incubated with 50 μM chrysin or solvent. The treatment with chrysin or solvent continued for 4 days every 24 hr, as described above. Twenty-four hr after the last medium change, the cell monolayers were washed twice with HBSS, scraped off the dishes into 0.15 M KCl in 10 mM sodium phosphate buffer (pH 7.4) (1 ml/dish). The cells were disrupted by sonication on ice (5 × 5 sec). The cell homogenate was either used for the catalytic assays or centrifuged at 9000g for 20 min at 4°C to obtain the supernatant fraction (S10). The residual concentrations of chrysin glucuronide and chrysin in the chrysin-pretreated cell homogenate were <0.5 μM, as determined by HPLC. The S10 fraction was further centrifuged at 100,000g for 60 min at 4°C. The microsomal pellets were resuspended in 300 μl of homogenization buffer with protease inhibitors (2 mM PMSF, 50 μg/ml antipain, 2 μg/ml aprotinin, 0.2 mg/ml benzamidine, 0.5 μg/ml leupeptin and 1 μg/ml pepstatin). Homogenates and microsomes were stored in aliquots at −80°C.

Determination of UGT Protein Levels by Immunoblotting

Microsomal samples were heated at 90°C for 5 min with an equal volume of sample buffer and loaded on a 12% SDS-polyacrylamide minigel together with molecular weight markers and positive and negative controls. After electrophoresis (16), the proteins were transferred to a nitrocellulose membrane (17). The membrane was blocked with 5% nonfat milk in 10 mM Tris/150 mM NaCl/0.05% Tween 20 (TBST) for 1 hr and incubated overnight at 4°C with anti-human UGT1A primary antibody in 5% nonfat milk in TBST at a 1:100 dilution. The blot was washed with 5% nonfat milk in TBST for 3 times 20 min and incubated with the secondary antibody, HRP-conjugated goat anti-rabbit IgG, at a 1:5000 dilution in 5% milk in TBST for 1 hr. After washing for 3 times 20 min with TBST, SuperSignal chemiluminescent substrate was added and the membrane was exposed to Hyperfilm ECL (Amersham, Piscataway, NJ) for 5 min.

Chrysin Glucuronidation by Caco-2 Cell Homogenates

Chrysin (1–20 μM) and 100 μl of pretreated or control Caco-2 cell homogenate (1.0 mg of protein) in 100 mM Tris.HC1 buffer (pH 7.4) with 5 mM $MgCl_2$ were prewarmed for 10 min at 37°C. The reactions were initiated by the addition of 0.5 mM UDPGA. The reaction mixtures (500 μl) were incubated at 37°C for 60 min. The samples were cooled on ice and centrifuged for 2 min at 10,000*g*. The supernatant was subjected to solid-phase extraction and HPLC analysis, as described above. Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation in Microsoft Excel.

Chronic Caco-2 Cell Flavonoid Treatment

Caco-2 cells were cultured as above and subcultured at a seeding density of 80,000 cells/cm^2 every 7 days. Fresh medium containing 10 μM chrysin, 10 μM quercetin or DMSO was added on days 2, 4 and 6 after each subculturing. After 5 weeks of pretreatment, chrysin metabolism experiments were done in 6-wells at 24 hr after the last flavonoid addition. The growth rate and viability were identical for flavonoid-treated and control cells.

Statistical analysis used student's unpaired *t*-test with a significance level of $p < 0.05$.

RESULTS

When the metabolism of chrysin by Caco-2 cells was analyzed by reversed-phase HPLC, a glucuronic acid and a sulfate conjugate were identified by MS and enzymatic techniques (11). After pretreatment of the cells for 4 days with 50 μM chrysin, the concentration of unchanged chrysin was reduced, whereas that of the glucuronic acid conjugate was markedly increased, with minimal change in the sulfate conjugate concentration. The results from 9 experiments, with and without pretreatment of Caco-2 cells with chrysin for 4 days are summarized in Fig. 2. Whereas the glucuronic acid conjugate increased 3.8-fold ($p < 0.0001$), the sulfate conjugate decreased 38% ($p < 0.01$). Although an increase in glucuronidation occurred as early as after a 1-day pretreatment, the maximum response to chrysin was reached after a 3- to 4-day pretreatment (Table I). The 30–50% reduction in the formation of the sulfate conjugate did not appear to be related to extensive consumption of the substrate by the glucuronidation pathway. When examining the influence of chrysin concentration on glucuronic acid conjugation, there appeared to be a linear increase with concentration up to 50 μM (Table II). Whereas low chrysin concentrations did not influence sulfate conjugation, 50 μM chrysin

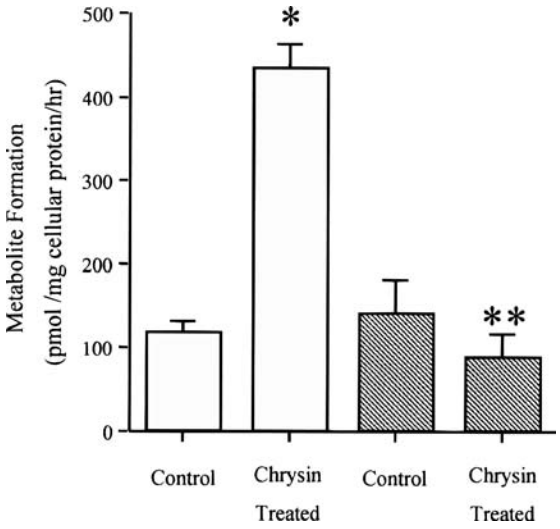

Fig. 2. Chrysin metabolism by Caco-2 cells after pretreatment with 50 μM chrysin for 4 days. Open bars designate chrysin glucuronide and hatched bars chrysin sulfate. Mean values ± SEM are shown (N = 9). * Significantly higher than Control, $p < 0.0001$. ** Significantly lower than Control, $p < 0.01$.

Table I. Effect of Pretreatment Time with Chrysin (50 μM) on the Formation of Chrysin Glucuronide and Sulfate

Pretreatment time (days)	Metabolite formation[a] (pmol/mg protein/hr)	
	Glucuronide	Sulfate
0	115 ± 11	109 ± 8
1	182 ± 15[b]	72 ± 8[b]
2	271 ± 49[b]	58 ± 16[b]
3	427 ± 66[b]	75 ± 7[b]
4	450 ± 20[b]	73 ± 6[b]

[a] Mean values ± SD (N = 3).
[b] Significantly different from 0 days ($p < 0.05$).

reduced this pathway similar to that in Table I. In separate experiments we also examined the effect of pretreatment with 50 μM genistein, an isoflavonoid, on chrysin metabolism. There was no effect on glucuronidation (126 ± 12 vs. 113 ± 12 pmol/mg protein/hr; treated vs. control), whereas sulfation was slightly decreased (226 ± 25 vs. 150 ± 19 pmol/mg protein/hr; $p < 0.05$) (n = 3).

Table II. Effect of Pretreatment Chrysin Concentration on the Formation of Chrysin Glucuronide and Sulfate

Pretreatment concentration (μM)	Metabolite formation[a] (pmol/mg protein/hr)	
	Glucuronide	Sulfate
0	115 ± 11	109 ± 8
5	135 ± 7	115 ± 5
10	187 ± 13[b]	108 ± 10
25	330 ± 46[b]	94 ± 15
50	445 ± 29[b]	60 ± 5[b]

[a] Mean values ± SD (N = 3).
[b] Significantly different from 0 days ($p < 0.05$).
Note: Incubations were done for four days.

We next examined the induction response in the Caco-2 cell homogenate after careful washing of the cells prior to the homogenization. The increase in glucuronidation after chrysin pretreatment was now as high as 14-fold compared to control (Fig. 3). Because of the relatively low level of chrysin glucuronidation in the homogenate of uninduced cells, accurate kinetics could not be determined. However, the apparent K_m value for glucuronidation of chrysin in the induced Caco-2 cell homogenate was estimated to be about 0.8 μM.

In the next series of experiments we determined whether chrysin pretreatment of Caco-2 cells produced an increase in UGT protein. For these experiments we prepared microsomes of pretreated and untreated cells and used an antibody, under development by Gentest (Woburn, MA), which detects all proteins in the UGT1A subfamily (Fig. 4). There was a substantial increase in the intensity of a ≈59 kDa band in the induced vs. uninduced control cells. Recombinant UGT1A6 protein served as a positive control, producing a band with a migration identical to that of the Caco-2 cell microsomes. Thus, chrysin pretreatment of Caco-2 cells resulted in markedly increased expression of one or more UGT1A isoforms. In contrast, experiments using an antibody specific for UGT1A6 failed to demonstrate induction of this isoform (data not shown).

Finally, we designed experiments in which Caco-2 cells were exposed to flavonoids for a period of 5 weeks. In addition to chrysin, we also studied quercetin, the most common flavonoid in our diet (18). We used flavonoid concentrations of only 10 μM in these experiments (Fig. 5). There was a more than 3-fold increase in the glucuronidation of chrysin in the intact Caco-2 cells after chrysin pretreatment, similar to the effect of 50 μM pretreatment in the acute experiments (Fig. 2). After quercetin pretreatment, there was an about 2-fold increase in the glucuronidation activity.

DISCUSSION

This is the first report on induction of members of the UGT family by dietary flavonoids in the human intestinal Caco-2 cells. This might have implications with respect to the oral

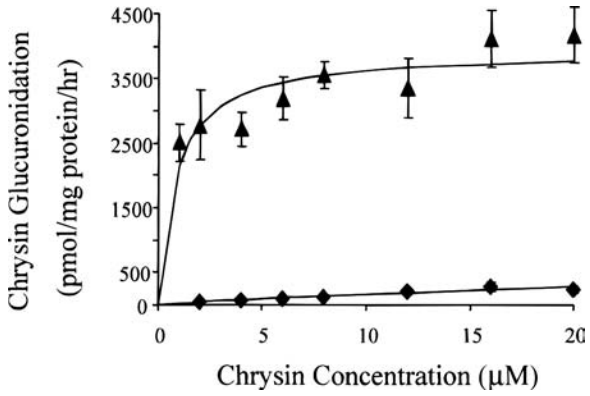

Fig. 3. Chrysin glucuronidation by Caco-2 cell homogenates prepared from untreated cells (♦) and cells pretreated with 50 μM chrysin for 4 days (▲). Mean values ± SEM from 3 experiments are shown. The error bars for the untreated cells are contained within the symbols. Data were fit to the Michaelis-Menten equation.

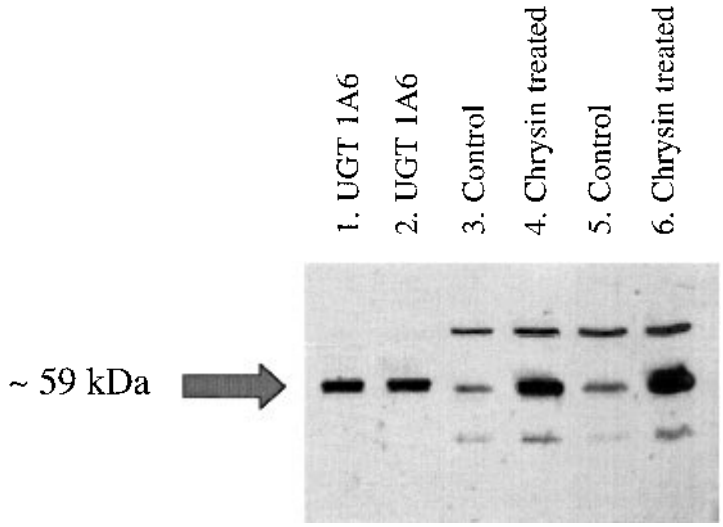

Fig. 4. Immunoblot analysis of Caco-2 cell microsomes from cells pretreated with 50 μM chrysin for 4 days (lanes 4 and 6, 50 and 100 μg loaded) and from untreated Control cells (lanes 3 and 5, 50 and 100 μg loaded). UGT1A6 microsomal protein (50 μg loaded) was used as a positive control in lanes 1 and 2.

bioavailability of carcinogens as well as other toxicological agents and commonly used drugs.

We have previously shown that the flavonoid chrysin is extensively metabolized by the Caco-2 cells through both glucuronidation and sulfation (4,11). This may seriously limit its therapeutic usefulness as a potent inhibitor of aromatase (19) and HIV activation (20). We now show that pretreatment of these human intestinal cells with chrysin markedly induces the metabolism of this flavonoid through the glucuronidation but not the sulfation pathway. The maximum induction response in the intact Caco-2 cells was a 3.8-fold increase in chrysin glucuronidation (Fig. 2) which was magnified to a 14-fold increase in the Caco-2 cell homogenate (Fig. 3). The lower apparent response in the intact cells may be due to high accumulation of substrate and product, as demonstrated in a previous study for quercetin (21), resulting in substrate/product inhibition. In contrast, both substrate and product in the homogenate were efficiently removed, as evidenced by HPLC. Maximum induction appeared to occur after 3–4 days of pretreatment and was linear with chrysin concentration in these short-term experiments. The inhibitory effect of chrysin on the sulfation pathway at 50 μM concentration (Tables I and II) appears similar to a previous observation for quercetin (22). The lack of induction by genistein points to interesting structure-activity relationships.

In order to test the effect of a chronic treatment schedule, we exposed the Caco-2 cells to either chrysin or quercetin for up to 5 weeks, using only 10 μM concentrations of each flavonoid. The induction response to chrysin was identical to that of the short-term exposure; quercetin, the most abundant flavonoid in our diet (18), was somewhat less effective. In terms of the flavonoid concentrations needed to produce a significant induction response, the 10–50 μM concentrations used in the present study are very likely to be achieved in the intestinal lumen after normal dietary exposure to these compounds (10).

A different measure of the induction response was obtained by SDS-PAGE of the microsomal proteins and immunoblotting. The use of a UGT1A subfamily-selective antibody clearly demonstrated increased levels of one or several isoforms of this group of enzymes (Fig. 4). Interestingly, experiments with a UGT1A6-specific antibody showed no evidence of induction. UGT1A6 (23–25) as well as UGT1A9 and UGT2B7 (25) have so far been identified in the Caco-2 cells.

Two important questions that need to be addressed are, first, which UGT isoforms are induced by chrysin and, second, what is the induction mechanism. Previous work has identified a number of UGT isoforms, which could potentially use chrysin

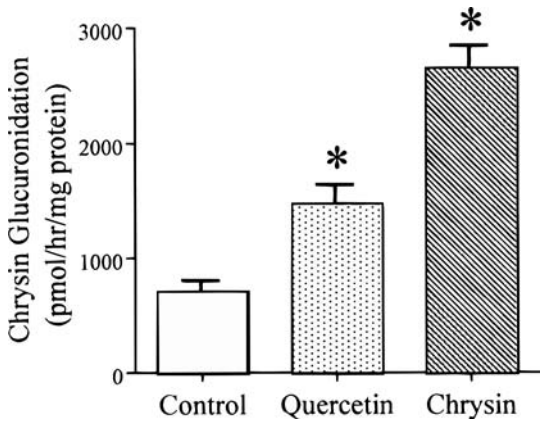

Fig. 5. Chrysin glucuronidation by intact Caco-2 cells after pretreatment of the cells with 10 μM quercetin or chrysin for 5 weeks. Medium containing solvent (Control), quercetin or chrysin was replaced every other day. After the last treatment, all cells were incubated with 50 μM chrysin for 24 hr for metabolite determinations. Mean values ± SEM are shown (N = 3–6). * Significantly higher than Control, $p < 0.05$.

and other flavonoids as substrates. These include UGT1A1 (26), UGT1A3 (27), UGT1A8 (28), UGT1A9 (29) and UGT2B15 (30,31). As recently shown by Strassburg *et al.* (32), UGT1A1, UGT1A3, UGT1A8 and UGT1A9 are all expressed in the normal human colon in addition to UGT1A4, UGT1A6 and UGT1A10.

Whereas UGT1A1, the isoform that metabolizes bilirubin, may be induced by phenobarbital (33,34), another UGT induction response is triggered by dioxin and polyaromatic hydrocarbons via an effect on the Ah-receptor. This has been shown to involve mainly UGT1A6 (24,25) but also UGT1A9 (25). A recent study indicated that the antioxidant *t*-butylhydroquinone induced UGT1A6, 1A9 and 2B7 (25). Where chrysin and quercetin induction fits in remains to be examined. Our preliminary observations suggesting that UGT1A6 may not be involved (see above) also suggest that the induction response produced by chrysin is different from that by dioxin and the polyaromatic hydrocarbons, i.e. may not involve the Ah receptor.

The practical implications of the observations in this study can only be speculated on as studies of the importance of the UGTs and their regulation in the human intestinal tract are in their infancy (32). These studies have suggested that the intestinal UGTs may play an important role in the detoxification of carcinogens and cytotoxic agents in general. These same enzymes may also limit the bioavailability of many therapeutically useful drugs.

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