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RAPID COMMUNICATION

Coordinate Induction by Antioxidants of UDP-glucuronosyltransferase UGT1A6 and the Apical Conjugate Export Pump MRP2 (multidrug resistance protein 2) in Caco-2 Cells

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ABSTRACT. Treatment of Caco-2 cells with the antioxidants quercetin or *t*-butylhydroquinone led to induced protein levels of UDP-glucuronosyltransferase UGT1A6 (ca. 3-fold over controls) and of the apical conjugate export pump multidrug resistance protein 2 (MRP2; 1.9-fold over controls). In contrast to UGT1A6, MRP2 (symbol ABCC2) was not inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Immunocytochemistry demonstrated that MRP2 was only expressed at the brush border domain of Caco-2 cell monolayers. The results indicate that UGT1A6 and MRP2 are coordinately induced by antioxidants, facilitating chemoprotection against phenolic toxins and excretion of conjugates into the intestinal lumen. BIOCHEM PHARMACOL **59**;5:467–470, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. human UGT1A6; MRP2; cMOAT; Caco-2 cells; antioxidants

Human UGT1A6 is a member of the superfamily of UGTs§ [1] and catalyses glucuronidation of a wide variety of planar phenols including neurotransmitters such as serotonin [2], drugs such as paracetamol [3], and phenolic metabolites of carcinogens such as benzo(*a*)pyrene [4, 5]. It is expressed in many tissues including human liver, intestine, kidney, lung, testes, ovary, and brain [2, 6, 7] and is probably involved in controlling cellular levels of endobiotics and in chemoprotection against phenolic xenobiotics. The resulting glucuronides are excreted from cells mostly by ATP-binding cassette (ABC) transporters of the MRP family [8–11]. Interestingly, the MRP2 isoform, also called cMOAT (canalicular multispecific organic anion transporter [9]; symbol ABCC2 [11]), appears to be expressed only at the apical membrane domain of epithelial cells, for example at the canalicular membrane of the hepatocyte, which facilitates secretion of conjugates into the bile [8].

Recently, Caco-2 cells have been found to be useful to study transcriptional activation of human UGT1A6 by aryl hydrocarbon (Ah) receptor agonists and by antioxidant-type inducers [12]. Both types of inducers have been shown to trigger distinct gene expression programs [13, 14]. In the present study, UGT1A6 and MRP2 protein were studied by immunoblot analysis after treatment of Caco-2 cells with the prototype inducers of these two expression programs, the potent Ah receptor agonist TCDD and the polyphenolic antioxidants quercetin or TBHQ.

MATERIALS AND METHODS

Chemicals and Reagents

Quercetin and TBHQ were purchased from Sigma and from Fluka, respectively. TCDD was obtained from Ökometric. Various alkaline phosphatase-conjugated antibodies, CDP-Star™ and I-Block™, were obtained from Tropix (Perkin Elmer Applied Biosystems).

Caco-2 Cell Cultures and Treatment

Caco-2 cell clone TC7 [15] was kindly provided by Dr. Alain Zweibaum (Institut National de la Santé et de la Recherche Médicale, Villejuif, France) and grown on 100 × 20 mm Falcon tissue culture dishes (Becton Dickinson) in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (heat-inactivated at 56° for 30 min), 25 mM glucose, and 1% non-essential amino acids (Life Technologies). The medium was changed daily. Preconfluent cells were treated with 10 nM TCDD, 80 μM TBHQ, and 50 and 100 μM quercetin dissolved in DMSO as indicated. Solvent controls contained 0.1% DMSO. Exposures were continued for 72 hr after which

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§ *Abbreviations:* UGT, UDP-glucuronosyltransferase; MRP, multidrug resistance protein; TBHQ, *t*-butylhydroquinone; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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cells were washed with 0.9% NaCl, harvested, and then stored at −80° prior to use.

Immunoblot Analysis

Caco-2 cell samples were quickly thawed and homogenised in 10 mM Tris–sucrose buffer pH 7.4, containing 1 mM phenylmethanesulphonyl fluoride, aprotinin, pepstatin A and leupeptin (50 μg/mL each), and 0.5 mM benzamidine using a Dounce homogenizer. Homogenate proteins, determined according to Lowry *et al.* [16], were denatured with Laemmli buffer containing SDS and 2-mercaptoethanol and heated at 95° for 10 min [17]. Proteins were separated by 10% (w/v) SDS–PAGE. Thereafter, proteins were transferred electrophoretically to Immobilon™ polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with a solution of 0.66% (w/v) I-Block™ in PBS and reacted with selected antibodies as described below. For quantitative immunoblot analysis of the isoform, the membranes were incubated overnight with selective sheep anti-human polyclonal UGT1A6 antibody (1:1000). In brief, the 120 N-terminal amino acids of the human UGT1A6 isoforms were expressed as a fusion protein with protein A in *Escherichia coli*. The fusion protein was then purified to homogeneity by affinity chromatography on an immunoglobulin G-Sepharose Fast Flow gel column and used to raise antibodies in sheep [18]. These antibodies selectively recognised UGT1A6 in human tissues as well as in recombinant V79 cells expressing individual UGT isoforms, exhibiting the same characteristics as rabbit anti-N-terminal UGT1A6 antibodies. MRP2 protein was determined using polyclonal antibody EAG5 raised against the C-terminal sequence of human MRP2 [19], which was kindly provided by Dr. Dietrich Keppler (Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Heidelberg). It was used at a dilution of 1:10,000. After extensive washing with I-Block™, immunocomplexes were detected by incubation for 90 min at room temperature with CDP-Star as chemoluminescence substrate and alkaline phosphatase-conjugated immunoglobulins (rabbit anti-sheep antibodies for UGT1A6 and goat anti-rabbit antibodies for MRP2). Imaging was performed with a CCD camera system and quantitative analysis using the program TINA 2.09 (Raytest). Several concentrations of homogenate proteins were applied to the gel, and linearity was obtained after background subtraction (not shown). Molecular weights for UGT1A6 [20] and MRP2 [11, 19], determined using a GIBCO stain protein ladder, were in accordance with the literature.

Immunocytochemical Studies

Cryostat sections of confluent Caco-2 cells were fixed in 4% phosphate-buffered paraformaldehyde for 5 min at 4° [15]. Thereafter, the sections were washed in PBS for 5 min and processed for indirect immunoperoxidase staining using 3,3′-diaminobenzidine tetrahydrochloride, as described [21]. The primary antibody against MRP2 was used at a dilution of 1:100; the secondary horseradish peroxidase-labelled swine anti-rabbit immunoglobulin G antibody (Dako) was used at 1:20 dilution. For villin staining, the antibody raised against mouse villin (described above) was used as primary antibody and horseradish peroxidase-labelled goat anti-mouse antibody (Sigma). Statistical analysis was performed using Student's *t*-test.

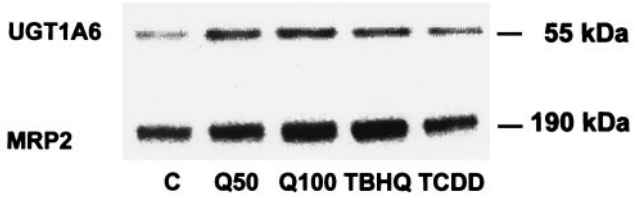

FIG. 1. Immunoblot analysis of UGT1A6 and MRP2. UGT1A6 and MRP2 induction by antioxidants in Caco-2 cells. Preconfluent Caco-2 cells were incubated for 72 hr with 50 and 100 μM quercetin (Q50 and Q100), 80 μM TBHQ, or 10 nM TCDD. Homogenate proteins were separated, blotted, and reacted with specific primary antibodies and alkaline phosphatase-labelled secondary antibodies as described in Materials and Methods. Each lane contained 10 μg protein. A representative blot of those analysed in Table 1 is shown.

RESULTS AND DISCUSSION

Availability of selective anti-human UGT1A6 antibodies prompted induction studies of UGT1A6 protein by immunoblot analysis after treatment of Caco-2 cells with TCDD or the antioxidants quercetin and TBHQ. It was shown that UGT1A6 protein was significantly increased after treatment with both types of inducers (Fig. 1 and Table 1). High standard deviations were probably due to the low avidity of the selective anti-UGT1A6 antibody. MRP2 has recently been characterised as an apical glucuronide export pump [11, 22]. In order to investigate its regulation, MRP2 protein was analysed in Caco-2 cells. It was shown for the first time that MRP2 and UGT1A6 are coordinately induced by antioxidants; however, in contrast to UGT1A6, MRP2 was not induced by TCDD. Immunocytochemical studies were carried out to clarify the localisation of MRP2 protein in Caco-2 cell monolayers. Staining for villin was used as marker for the brush border membrane domain [15,

TABLE 1. Immunoblot analysis of UGT1A6 and MRP2 induction by antioxidants or by TCDD

Treatment	UGT1A6 (% of controls)*	MRP2 (% of controls)*
Quercetin (50 μM)	235 ± 68†	133 ± 5†
Quercetin (100 μM)	333 ± 172†	172 ± 19†
TBHQ (80 μM)	303 ± 125†	186 ± 38†
TCDD (10 nM)	280 ± 145†	110 ± 24

* Quantification is based on mg cell protein. Data represent means ± SD of 3 independent induction experiments (each analysed twice). 100% represents protein levels in solvent controls containing DMSO.

† $P < 0.05$.

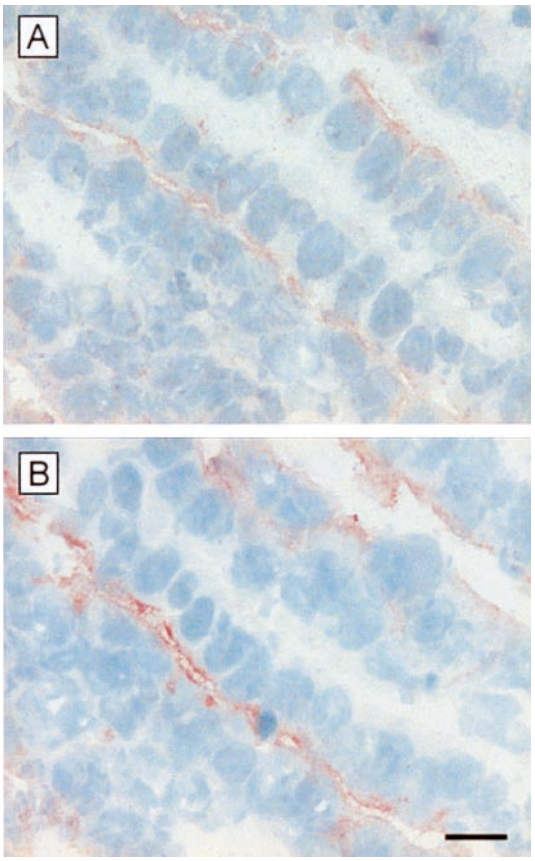

FIG. 2. Immunochemical staining of MRP2 (A) in comparison with villin (B) in Caco-2 cell monolayers. Cryostat sections of 50 μM quercetin-treated cells were reacted with anti-MRP2 and anti-villin as first antibody and with horseradish peroxidase-labelled second antibodies. The bar represents 50 μm.

23]. It was found that MRP2 was only present at the brush border domain of Caco-2 cell monolayers (Fig. 2).

The present study demonstrates for the first time that the organic anion export pump MRP2 is inducible by antioxidant-type inducers in Caco-2 cells, albeit to a small extent. In contrast to UGT1A6, MRP2 is not inducible by TCDD. Several UGTs (UGT1A6, UGT1A9, and UGT2B7) appear to be induced by antioxidants, as shown previously by RT–PCR (reverse transcriptase–polymerase chain reaction) and UGT activity studies [12]. These studies showed ca. 2-fold increases in UGT1A6 mRNA following treatment with 80 μM TBHQ or 10 nM TCDD. UGT activity (4-methylumbelliferone as substrate) was also increased 2.1- and 1.6-fold by TBHQ and TCDD, respectively [12]. 4-Methylumbelliferone is a substrate of several UGTs. Therefore, UGT1A6 induction by TCDD and antioxidants was substantiated in the present study by determination of the enzyme protein. MRP2 was shown to be expressed only at the brush border domain of Caco-2 cells (Fig. 2). It has been shown to be functionally active in these cells in studies of glucuronide transport of the flavonoid chrysin [24]. Glucuronides are formed by UGTs at the lumenal part of endoplasmic reticulum membranes and have to be transported through the membrane to the cytosol before being exported from cells by MRP2. However, carrier proteins responsible for glucuronide transport from the endoplasmic reticulum lumen to the cytoplasm have not yet been identified. Apical localisation of MRP2 suggests that glucuronides are mainly transported out of the enterocyte to the intestinal lumen [24]. Therefore, coordinate induction of UGT1A6 and MRP2 may have a number of pharmacological and toxicological implications. It may explain chemoprotection against dietary phenolic compounds which are conjugated directly by the intestinal mucosa. On the other hand, hydrolysis of glucuronides by bacterial β-glucuronidases in the intestinal lumen and reabsorption of the aglycones may lead to futile cycles. For example, these cycles may explain in part gastrointestinal toxicity (diarrhoea) of the potent chemotherapeutic and camptothecin derivative, irinotecan. Irinotecan has been shown to be rapidly converted in liver and intestine to its glucuronide and excreted via MRP2 [25].

In conclusion, induction studies with Caco-2 cells clearly indicate coordinate induction of UGT1A6 and MRP2 by antioxidant-type inducers (but not by TCDD), facilitating secretion of glucuronides into the intestinal lumen. This finding may be important in chemoprotection against phenolic compounds.

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